PROTECTIVE ROLES OF miR-29a AND miR-18a IN LIVER FIBROSIS

By

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Abstract

The research described in this dissertation highlights the need for clinical therapeutics for fibrosis, targeting fibrotic mechanisms and how miRNAs can be powerful modulators of fibrosis. miRNAs are 22 nucleotide long, post-transcriptional regulators of gene expression. We profiled miRNA expression changes in two different models of liver fibrosis and identified two different miRNAs which showed differential expression in fibrotic livers.

miR-29 family targets many proteins in the extracellular matrix and is downregulated during fibrosis in many organs. We developed a viral vector based delivery of miR-29 to replace lost miR-29 during fibrosis. Successful prevention and reversal of ongoing hepatotoxin mediated fibrosis was seen with hepatocyte-specific expression of the viral vector, illustrating the powerful anti-fibrotic role of miR-29. Our research supports and encourages industry interest in developing miR-29 based drugs, which takes us one step closer to alleviating distress of patients in the clinic.

Alcohol mediated fibrosis is a major health problem in the world and is the major cause contributing to the increased need for liver transplantation. We studied miR-18a, which is increased in alcohol and hepatotoxin mediated liver fibrosis. miR-18a expression is localized to cells in the same region as fibroblasts, which are the key responders in fibrosis. This miRNA targets components of TGF-β signaling pathway and affects pro-fibrotic processes such as fibroblast migration and collagen production. miR-18a is downregulated by TGF-β, illustrating that TGF-β downregulates its own negative

regulator, fine-tuning signaling activity. Combined, these results strongly suggest a protective role of miR-18a during liver fibrosis.

This work establishes a large body of knowledge about miRNAs and their roles in regulating fibrosis. We identified cell type specific expression and function of miRNAs within the liver that further confirms the need to look not only at whole tissue expression but also in different individual cells. We have highlighted the role of miRNAs in regulating and therapeutically treating liver fibrosis and hope that clinical drugs of miRNAs would not be too far in the future.

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Preface

I am profoundly grateful for the opportunity that I've had to conduct this research, and for the many special people that I've met along the way. First and foremost I thank and acknowledge my thesis advisor and mentor, Daniel Warren. He has taught me how to approach problems in a systematic way and analyze data impartially. In addition, Dan has also taught me qualities such as patience, pragmatism and diplomacy which are useful in all facets of life.

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1. CHAPTER 1. INTRODUCTION

1.1 Fibrosis is a progressive disease affecting multiple organs

Fibrosis (from Latin *fiber* = filament) is defined as an unchecked, excessive deposition of extracellular matrix (ECM) components in organs and tissues as a consequence of proliferation and activation of fibroblasts. The process of fibrosis begins as a wound-healing reaction but sustained injury leads to the excessive deposition of ECM proteins, including collagens and fibrillins. Wound healing response to injury in tissues initially involves production of immunogenic factors and other chemokines. In all tissues, fibroblasts are the main cells that proliferate and secrete connective tissue components (ECM proteins as seen in scars). Under normal conditions, there is regeneration of damaged tissues, wound contraction and resolution of scarring. When there is chronic, sustained injury, fibroblasts migrate and result in uncontrolled production and deposition of ECM proteins. This can result in progressive substitution of parenchyma by scar tissue and changes in organ architecture and/or function.

In the human body, there are many different insults – both primary (genetic or idiopathic) and secondary (external factor induced injury) that induce fibrosis in multiple tissues. The shared pathways of wound healing and fibrosis is conserved across many organs and results in varied clinical diseases (table 1.1). External factors such as drugs, infections and auto-immune responses can also lead to fibrosis of the heart, lung, kidney and liver.

The clinical burden of fibrosis is huge and increasing because of increased injury to many organs and lack of current therapies to treat fibrosis. Nearly 45% of all

deaths in the developed world are attributed to some type of chronic fibro proliferative disease (Wynn, 2007).

1.2 Liver fibrosis has a high clinical burden

Chronic liver disease and cirrhosis (the most severe fibrotic state of liver) can arise spontaneously or because of external injuries (Table 1.2). A Centers for Disease Control and prevention (CDC) report shows that chronic liver disease and cirrhosis was the 12^{th} most common cause of death in the USA in 2010 and again in 2011, with increasing incidence every year (Murphy, 2013).

Liver fibrosis is markedly different from other organs, because the early stages of fibrosis are reversible due to the regenerative capacity of the organ. When the injury stimulus is removed, the balance between the synthesis and catabolism of ECM production shifts back to equilibrium, and there is resolution of fibrotic scarring. Hepatic fibrosis can take up to a few decades to develop to a symptomatic state (Schuppan and Afdal, 2008). Patients may be tested for viral DNA or liver enzyme levels, based on other symptoms or annual checkups, leading to incidental diagnosis of liver fibrosis. Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) are hepatic enzymes that are released into the bloodstream from damaged hepatocytes. The predictive value of the AST/ALT ratio has been validated in many different etiologies of fibrosis in clinical patients (Haukeland, 2008). Definitive evidence is usually provided by biopsy or liver imaging methods. Only severely cirrhotic patients present with outwardly visible symptoms such as increased portal hypertension, jaundiced appearance, ascites in the abdomen, splenomegaly etc.

There are no FDA approved therapies for fibrosis, even if the diagnosis is positive, removing offending insults (for e.g., anti-viral medication) or promoting lifestyle changes is the current treatment regimen. Suggested lifestyle changes include reduced drinking for all viral infected and alcoholic liver fibrotic patients. Once the disease has progressed to a severe, irreversible state, the only option is to perform a liver transplantation. The U.S. Department of Health and Human services estimated that, in 2011, 5,805 adult liver transplants were performed in the United States, while 2,456 patients died while on the waiting list. This illustrates the non-availability of therapies to delay or ablate ongoing fibrosis and the acute shortage of available organs for transplantation.

The main liver diseases in developed nations such as USA, is viral hepatitis (B, C, D, and E), alcoholic liver disease, and NAFLD. This is in contrast to developing nations where infectious and parasitic diseases still play a major role in liver damage, in addition to hepatitis virus and alcoholism. Viral infection and alcoholic liver damage increases the risk of developing fibrosis in a newly transplanted liver in a short time, further adding to the burden of organ transplantation.

Cirrhosis is the major risk factor for hepatocellular carcinoma (HCC). El-Sarag *et al.*, in 2007 assessed that 10,000 deaths occur each year from HCC alone. In summary, the fibrotic diseases of the liver and its complications are cumulative over many years and have a heavy clinical burden because of the lack of approved anti-fibrotic therapy reversing early-stage fibrosis.

1.3 Fibroblasts are the major cells and key responders in fibrotic diseases

The initial injury to an organ is first sensed by epithelial or endothelial cells which release inflammatory mediators helping to recruit leukocytes to the site of injury. Activated leukocytes (white blood cells) secrete pro-fibrotic cytokines such as TGF-β and interleukins. This further activates epithelial or endothelial cells and helps recruit more cells of native and adaptive immunity, increasing cytokine secretion. Shortly after, fibroblasts become activated, proliferate and migrate to the site of injury, express smooth muscle actin (α-SMA) and become myofibroblasts. Many different cells can contribute to this pool of myofibroblasts, including immature fibrocytes, epithelial cells displaying fibroblast characteristics (through the epithelial-mesenchymal transition, EMT), endothelial to mesenchymal transitioned (EndoMT) cells, bone marrow stem cells, and tissue specific cells such as hepatic stellate cells in the liver (Lebleu, 2013).

Chronic injury with persistent inflammation, tissue necrosis and other changes alters the secretion of ECM proteins by the myofibroblast cells, leading to fibrosis. ECM proteins include glycosaminoglycan and fibrous proteins such as collagens, elastin and fibronectin. The ECM synthesized during fibrosis is different in both composition and quantity from the normal physiological matrix. In liver fibrosis, type I collagen increases by a large amount, with some increase in type IV collagens in and around portal areas (Consolo et al., 2009). This increase in collagen is mostly transcriptional, mediated by TGF-β (Armanderiz-Borunda, 1992) but post-transcriptional control of collagen is necessary for formation of collagen fibrils and extracellular secretion.

ECM homeostasis is maintained by the balance between synthesis/assembly of fibrous proteins and degradation of existing fibrils. Two groups of proteins, MMPs (matrix metalloproteinase) and TIMPs (Tissue inhibitor of matrix metalloproteinase),

control the rate of digestion of the ECM. MMPs cleave protein chains of collagens while TIMPs are the endogenous inhibitors of MMPs, which function not by blocking protease activity but by modulating MMP function. Different MMPs target different ECM proteins, either in the normal basement membrane matrix, or the fibrillar liver collagens in disease (Benyon and Arthur, 2001). TIMPs are increased in fibrosis; they may play complex roles by targeting only those MMPs that degrade normal basement membrane collagen. The ratio of MMP/TIMP and the exact composition of elevated MMPs and TIMPs can be a diagnostic indicator of fibrosis (Lichtinghagen, 2000).

The excessive deposition of ECM, and the increase in inflammatory/pro-fibrotic mediators leads to changes in architecture of the organ, and pathological loss of function. Fibrosis results in myocardial stiffness and dysfunction in the heart (Weber and Brilla, 1991), breakdown of the glomerular filtration barrier, hypertension in the kidney (Horl, 2000), interstitial thickening of alveolar wall and alveolar collapse in the lung (Crouch, 1990), portal hypertension or jaundice in the liver (Kew, 1971) and results in cardiac, renal, lung and liver failure respectively.

1.4 Overview of different fibrogenic pathways in fibrosis

It has been formulated that key pathways and molecules in fibrosis can be divided into core and regulatory pathways. Core pathways are broadly active in many organs and species and thus probably necessary for modulating fibrosis, and regulatory pathways only guide the core pathways and are likely to vary between organs, species and individuals (Mehal, 2011). Major core pathways in fibrosis include that of cytokines such as Transforming growth factor-β (TGF-β) , growth factors such as platelet-derived, and

connective tissue growth factors (PDGF, CTGF), and immunogenic pathways including interleukins and other chemokines.

TGF-β superfamily proteins regulate many different cellular processes, such as embryonic development, wound healing, immune response, and cell cycle control (Mccartney-Francis, 1998). Perturbation of this pathway can lead to many different diseases including cancer, fibrosis, hypertrophy and inflammation (Akhurst and Hata, 2012). TGF-β is the major pro-fibrotic cytokine responsible for initiating and mediating fibrosis and is synthesized by a wide variety of cells including macrophages, neutrophils and fibroblasts. It also mediates activation and differentiation of fibroblasts to myofibroblasts, and their migration to site of injury (Evans, 2003) and regulates EMT (epithelial to mesenchymal transition). Importantly, TGF-β induces fibroblasts to synthesize and contract ECM (LeRoy, 1990). Transcriptional up regulation of collagen by both SP1 and Smad mediated mechanisms is widely conserved and active in many different organs during fibrosis (Ghosh, 2000; Nehls, 1992). Since TGF-β targets migration, differentiation and ECM production of fibroblasts, it has been established to be pro-fibrotic in animal models and in human disease, including lung (Khalil and Greenberg, 1991), Skin (Trojanowska, 2009), kidney (Yamamoto, 1996), and Liver (Flisiak, 2000).

CTGF, a TGF-β induced cytokine, promotes fibroblast proliferation, matrix production, and granulation tissue formation (Leask and Abraham, 2004). It also promotes cell adhesion and migration in smooth muscle cells and fibroblasts (Crean, 2002). CTGF has been shown to act as a downstream effector of the pro-fibrotic effects of TGF-β under certain conditions (Grotendorst, 1997) and also as an independent

regulator of fibrotic phenotypes (Gore-Hyer, 2002). CTGF is coordinately expressed with TGF-β in every fibrotic disorder examined (Grotendorst, 1997), and is increased in animal models and patients in fibrosis of the heart (Koitabashi, 2007), lung (Ponticos, 2009) and other organs.

PDGF is another growth factor involved in regulating cell growth and division and is most well-known for modulating angiogenesis. PDGF is a potent mitogen for cells of mesenchymal origin, and a chemo-attractant for fibroblasts. PDGF is secreted by alveolar macrophages and vascular smooth muscle cells in the lung (Vignaud, 1991), hepatic macrophages (also known as Kuppfer cells), stellate cells and endothelial cells in the liver (Friedman and Arthur, 1989), smooth muscle and mesangial cells in the kidney (Bhandari, 1994) and in skin macrophages (Gay, 1989). PDGF ligand functions mainly through the MAPK pathways by binding to its receptors in fibroblasts and mediating enhanced replication, migration and in certain conditions, even ECM protein deposition at sites of tissue injury (Bonner, 2004).

Inflammatory-immunologic responses to an injury happens at the early stages, preceding the pro-fibrotic processes. The varying contributions of innate and adaptive immunity to fibrosis has not been delineated yet. Interleukins fall in both proinflammatory (IFN-γ and IL-2) and anti-inflammatory (IL-4, IL-10, and IL-13) cytokine classes. IL-10 controls the balance between MMPs and TIMPs, both crucial for degradation of ECM proteins (Ghosh, 2013). Normally, a massive infiltration of immune cells occurs in fibrotic tissues, secreting the initial pro-fibrotic molecules, including TGF-β. For example, in autoimmune systemic sclerosis, parasitic schistosomiasis induced fibrosis or hepatitis B virus mediated infection of the liver, the lymphoid infiltrate attacks

the affected cells (Wick, 2013). Immune response provides the continued insult during an injury and hence may be useful therapeutic targets in the clinic. Supporting this, interferon (IFN -γ1b) has been shown to inhibit fibroblast proliferation and collagen synthesis and has been associated with reduced mortality in IPF patients (Bajwa, 2005).

1.5 Regulation of fibrosis by microRNAs

MicroRNAs (miRNAs) are a large family of post-transcriptional regulators of gene expression, which function by base-pairing to complementary sequence in 3' UTR (untranslated region) of mRNAs and suppressing translation or promoting cleavage of the mRNA. Binding is mostly regulated by complementarity between seed sequence of ~7 nucleotides of a miRNA and its target mRNA, and each miRNA can potentially target hundreds of genes, influencing diverse regulatory pathways- both in normal physiology and disease.

miRNA biogenesis involves multiple processing steps (Winter, 2009) and final mature miRNA expression can be regulated at any one of these steps. miRNAs are transcribed from exonic, intronic or intergenic locus by pol II polymerase to form primary miRNAs (pri-miR), which can encode single or multiple miRNAs. The pri-miR fold into a hairpin with stem-loop structures and the hairpin is cleaved by endonuclease Drosha into a ~80 bp precursor miRNA (pre-miR) hairpin. Pre-miRs are exported out of the nucleus by exportin 5, to the cytoplasm, where the loop is cleaved by another endonuclease Dicer to form a 22 bp imperfect miRNA duplex. Usually, only one of the strands of this duplex is chosen as the mature miRNA, and is incorporated into the RNA-induced silencing complex (RISC), where it binds to its target mRNA 3' UTR sequence

and induces cleavage (if perfect complementarity) or translational suppression (in case of imperfect binding). Both strands of the miRNA duplex may also be active, leading to increased number and more diverse targets (Almeida, 2012; Rakoczy, 2013).

miRNAs have been implicated in many fibrotic disorders, both in animal models and diseased humans, in various organs (Figure 1.1). As of now, a total of 46 miRNAs have been found to be dysregulated in fibrosis but only a few have been revealed to be important for fibrosis in multiple organs.

miRNAs target key pathways during fibrosis and the many fibrotic miRNAs can be split into three main groups- those targeting

- Downstream ECM synthesis, organization, degradation
- Cell physiological activities such as proliferation, apoptosis, migration
- TGF-β/ CTGF mediated signaling and phenotypic changes

These core pathways, active in multiple organs and multiple fibrotic disorders, is also targeted by miRNAs to enhance or protect from fibrosis. This section reviews currently known fibrosis associated miRNAs.

Modulation of ECM accumulation by miRNAs

miR-29 members have been established to be the key regulator of ECM protein synthesis; their direct targets include many collagens, elastin, and fibrillins. The role of miR-29 as an anti-fibrotic regulator has been shown in multiple fibrotic diseases and organs and species. In Chapter 2, downregulation of miR-29 in the course of fibrosis and therapeutic benefits of maintenance of miR-29 using a viral delivery method will be discussed.

NF-κB is a key regulator of inflammation and stress response in the heart, resulting in cardiac hypertrophy and fibrosis. miR-26a is down regulated by Angiotensin II, a hormone regulating blood pressure and CTGF production, through binding of NF-κB to miR-26a promoter. miR-26a in turn represses its direct targets CTGF and collagen I and functions as a negative feedback regulator of NF-κB activation in cardiac fibrosis (Wei, 2013). miR-199b, in contrast, plays a pro-fibrotic role in liver and heart. The induction effect of miR-199b on collagen, TIMP, and MMP was shown in liver fibrosis, both in hepatotoxic liver injury in mice and in human liver (Murakami, 2011). miR-199b is also in a feed forward loop with NFAT (nuclear factor of activated T cells) protein, leading to cardiac fibrosis and hypertrophy (da Costa Martins, 2010).

Fibrosis in the kidney can occur due to complications from diabetes. miR-377 has been shown to be increased in the mesangial cells, the fibroblasts of the kidney, in diabetic nephropathy. miR-377 directly targeted p21-activated kinase 1 (PAK1), and superoxide dismutases 1 and 2 (SOD1, 2) leading to increased fibronectin buildup (Wang, 2008). Additionally in the kidney, downregulation of miR-449a/b during hypoxia decreases the rate of ECM degradation and induced fibrosis. miR-449 a/b targets PAI-1 (Plasminogen Activator Inhibitor-1), which blocks conversion of pro-MMPs to MMPs by inhibiting the serine protease PA (plasminogen activator) (Muth, 2010).

The basic structural unit of collagen is a triple-helix, which is further post-translationally modified and secreted as cross-linked, insoluble fibrils into the extracellular medium (Birk, 1989). The final concentration of collagens can be controlled at the rate of transcription to any of the post-translational steps or even secretion outside of the cell. miR-122 targets one of the genes involved in modification of the prolines in

the pro-collagen triple helix, the enzyme prolyl 4-hydroxylase subunit alpha-1 (P4HA1), resulting in decreased stability of the helix and degradation of collagen. miR-122 was decreased in mice livers treated with the potent hepatotoxin carbon tetrachloride, suggesting that it could be used as a therapeutic molecule in liver fibrosis (Li, 2013).

Alteration of cell physiological activities by miRNAs

Many miRNAs influence cell physiology during fibrosis, by changing proliferation, apoptosis, or migration, especially in fibroblasts, the main cells producing ECM. In fibrosis, the liver specific fibroblasts, HSC lose their characteristic retinoid droplets, become contractile and begin synthesizing ECM components. These "activated" HSC proliferate and migrate to the site of injury and alter the rate of ECM synthesis and degradation (Friedman, 2008). miR-27a/b target the vitamin A binding downstream transcriptional activator RXR-α (retinoid X receptor-alpha) and accelerate the conversion of HSCs to an activated phenotype. miR-27a/b are increased in activated in-vitro HSCs and increase the proliferation rate, presumably due to RXR-α transcriptional effects (Ji, 2009). Proliferation of HSCs is also regulated by miR-150 and miR-194, which directly target C-myb and rac1 respectively. C-myb is a transcription factor and rac1 mediates production of superoxide anion, both shown to be involved in the proliferation of HSC and development of fibrosis (Kitada, 1997; Sundaresan, 1996). Accordingly, both miR-150 and miR-194 were found to be down regulated in a bile duct ligation induced fibrosis in rats (Venugopal, 2010).

Chemotaxis of fibroblasts towards site of injury precedes the exaggerated deposition of ECM components in an organ. miR- 335 directly down regulated tenascin-C (TNC), a glycoprotein in the ECM regulating cell migration (Chen, 2011). Activated

stellate cells in cell culture showed low miR-335 and high TNC, and increasing miR-335 reverted this phenotype and also reduced collagen I and α-SMA. Often, fibrosis is a secondary development, and many different cell types need to respond appropriately to bring back the organ to normal. In the heart, miR-132 was constitutively expressed and secreted by protective pericyte progenitor cells in the saphenous vein (SVP).These mural cells wrap around endothelial cells along vessels and provide protective immune and other physiological signals in paracrine fashion to endothelial cells (Chen, 2013). miR-132 stimulated angiogenesis in the infarcted heart and reduced myofibroblast differentiation by silencing RAS-GAP (Ras-GTPase activating protein) and MeCP2 (Methyl-CpG-binding protein 2). When these miR-132 secreting SVP cells were transplanted into MI hearts, they attenuated apoptosis and fibrosis of the interstitium (Katare, 2011).

Apoptosis of fibroblasts has been proposed to play an important role in reversing myofibroblastic accumulation and eliminating fibrosis (Friedman, 2008). miR-15b and miR-16 were found to be downregulated in activated rat HSCs in-vitro; restoring their expression led to disruption of Bcl-2, a potent apoptosis inhibitor. Thus, downstream apoptosis inducing caspases were increased and resulted in elevated cell death in activated stellate cells (Guo, 2009). In diabetic nephropathic kidneys of mice, miR-216a and miR-217 function in a similar way by directly repressing PTEN (phosphatase and tensin homologue), a major tumor suppressor gene controlling cell cycle. The co-transcribed miRNAs are upregulated by TGF-β and modulate cell survival by increasing Akt signaling. This allows further fibrotic steps to occur uninhibited such as hypertrophy and ECM production (Kato, 2009).

miRNA regulation of TGF-β and CTGF signaling

TGF-β is a central mediator of fibrosis in all known organs and species. Therefore, miRNAs targeting this central pathway signaling, either through repressing ligand and receptors or downstream signaling proteins, modulate fibrosis profoundly. TGF-β can signal through many different pathways; the core or canonical one is through smad proteins (Lindsay and Dietz, 2011). TGF-β superfamily ligand binding to receptor (type II) leads to recruitment and phosphorylation of receptor (type I), which in turn phosphorylates receptor regulated smads (R-smads such as smad2, 3) which can now bind to co-stimulatory smad (co-smad, Smad4). This complex moves to the nucleus and act as transcription factors regulating gene expression. TGF-β can also signal through other non-canonical pathways including MAPK, JNK and p38 group of kinases. Some miRNAs directly downregulate the ligands TGF-β1 (miR-122, miR-133) or TGF-β2 (miR-141, 200a). miR-122 is decreased in aortic valve stenosis, correlating with increased TGF-β1, resulting in severely fibrotic phenotype in the myocardium (Beaumont, 2013). miR-141 and miR-200a share the same seed sequence and both target TGF-β2, resulting in elevated ECM gene expression and EMT. As expected, both the miRNAs are decreased in two models of kidney fibrosis, diabetic and adenine mediated (Wang, 2011). miR-590, in contrast, targets TGFBR2, and is down regulated in a nicotine-induced atrial fibrillation model in dogs, increasing collagen production in the heart (Shan, 2009). miR-133, which targets TGF-β1 and CTGF, is also decreased in the same canine atrial remodeling and also in two other models of cardiac failure- a hypertension induced heart failure in rats and aortic binding in mice. Importantly, miR-133 was also decreased in early stages of

aortic stenosis in human patients, indicating that it may be used as a diagnostic in a clinical setting (Duisters, 2009).

Another way to inhibit TGF-β signaling is to target genes in the downstream signaling pathways (E.g.: smads). (He, 2012) showed that smad4 (an in-silico target of miR-146a) protein expression was decreased when miR-146a was overexpressed in stellate cells in-vitro. miR-146a was downregulated in hepatoxin induced liver fibrosis in rats and overexpressing it corrected the phenotypes of fibrosis in-vitro, such as decreased HSC apoptosis, increased α-SMA expression, and increased TGF-β induced proliferation. miR-21 is a major pro-fibrotic miRNA, which aids in increasing TGF-β signaling in lung, heart and kidney. miR-21 has been shown to inhibit the protective smad7 (TGFBR1 antagonist) in lung fibrosis (Liu, 2010), and spry-1 (receptor tyrosine kinase inhibitor) augmenting TGF-β induced MAPK-ERK signaling, in heart fibrosis (Thum et al., 2008). TGF-β also induces miR-21 via a smad3 dependent mechanism in an animal model of UUO (unilateral urethral obstruction affecting kidneys) (Zhong, 2011) and this upregulation was also seen in the serum of clinical patients with kidney fibrosis (Glowacki, 2013), making miR-21 a novel blood marker of fibrosis in the kidney. In the heart, miR-21 is upregulated in border zones of myocardial infarction (MI), and down regulates its target TGFBR3, negative regulator of TGF-β, increasing collagen production (Liang, 2013).

miR 17-92 family members target many different proteins in TGF-β signaling including TGFBR2, CTGF, smad2, smad4 and significantly inhibit fibrosis in many organs. Chapter 3 will focus on understanding the role of one of the members of this family, miR-18a and the protective role it plays in fibrosis.

Epithelial to mesenchymal transition is reported to be one of the major pathways for the increase in myofibroblasts during fibrosis. TGF-β is also known to be key regulator of this process, inducing core regulators SNAIL1,2 and ZEB1,2 (zinc-finger E-box binding factor 1,2) (Massague, 2012). ZEB family induces EMT by transcriptionally repressing epithelial genes including E-cadherin, tight and gap junction proteins along the epithelial cell-cell lining (Vandewaale, 2005). miR-192 and miR-215 (both have the same seed sequence) miRNAs directly target ZEB2, thereby increasing E-cadherin and also collagen in cells of the diabetic kidney (Kato, 2007). TGF-β and CTGF play opposing roles in regulating miR-192/215; CTGF induces these miRNAs whereas TGF-β suppresses their expression (Wang, 2010). miR-200b also represses ZEB1/2 and prevents EMT in renal tubulointerstitial fibrosis, induced by UUO in mice (Oba S, 2010).

1.6 Investigating the protective roles of miR-29 and miR-18a in liver fibrosis

Liver fibrosis is one of the few fibrotic diseases where the slow progression of the disease, regenerative nature of the organ and ease of delivery of drugs through the portal circulation, makes it feasible to study and therapeutically treat fibrosis. Current therapeutics do not target any of the common processes of fibrosis; existing ones target the cause of liver damage (e.g. anti-viral medicine). miRNAs are potent post-transcriptional regulators of gene expression and many play a role in manipulating core pathways such as TGF-β signaling or ECM synthesis. In the next few chapters, we will identify a novel method of delivery of miR-29 to correct fibrosis in mice and recognize a key anti-fibrotic miRNA, miR-18a in hepatotoxin exacerbated alcoholic liver fibrosis in rats.

1.7 Tables: Chapter 1

Table 1-1. Fibrosis occurs in many different organs/tissues

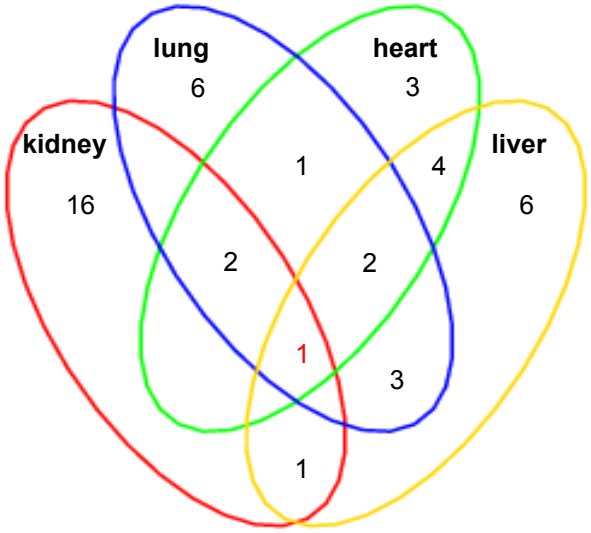
Organ/Tissue	Example
Liver	• Cirrhosis
Lung	• Idiopathic Pulmonary Fibrosis (IPF)
Heart	• Endomyocardial fibrosis • Atrial fibrosis • Myocardial infarction
Skin	• Scleroderma • keloid
Systemic	• Systemic sclerosis • Cystic fibrosis
Intestine	• Crohn's disease
Bone marrow	• Myelofibrosis
Soft tissue in joints	• Arthrofibrosis

Table 1-2: Common etiologies of liver fibrosis in the clinic

Cause	**example**
Genetic/metabolic	• Wilson's disease
Autoimmune	• Primary biliary cirrhosis • Primary sclerosing cholangitis • Autoimmune hepatitis
Virus	• Chronic viral hepatitis
Lifestyle	• Alcoholic liver disease • Non-alcoholic fatty liver disease • Drug-induced fibrosis
Environment	• Schistosomiasis (parasite induced) • Toxic environmental exposure
others	• Transplant rejection

1.8 Figures: Chapter 1

Figure 1-1. microRNAs implicated in fibrosis in 2 or more organs



This Euler plot diagrammatically presents the number of miRNAs implicated in regulating fibrosis in each of the four organs. Those that have been shown to modulate fibrosis in 2 or more organs are more likely to be those of "core" miRNAs and therapeutically useful in the clinic. For example, there is only one miRNA known to be implicated in all four organs (the 1 in red), miR-29 family.

2. CHAPTER 2. THERAPEUTIC DELIVERY OF miR-29a PREVENTS AND REVERSES LIVER FIBROSIS

2.1 Introduction

Liver is a vital organ performing many specialized and diverse functions, such as detoxification, bile production for lipid digestion, gluconeogenesis, and cholesterol and hormone synthesis. Hepatocytes, the parenchymal cells make up 80% of liver by volume. Non-parenchymal cells such as endothelial cells, Kupffer cells (liver macrophages), and hepatic stellate cells (resident fibroblasts of the liver) line the sinusoids and make up about 6.5% by volume (Kmiec, 2001). Blood flow into the liver is from two different sources: the hepatic artery carries oxygenated blood from the aorta and the hepatic vein carries venous blood from gastrointestinal tract, spleen and pancreas. This blood flows through the liver sinusoids lining the hepatocytes and drains into the central vein of each lobule. A distinctive arrangement of branches of the hepatic artery, hepatic vein and bile duct is called portal triad in the liver. Lobules are smaller units of the liver defined at the histological scale, and they can be viewed from different perspectives. A classical lobule can be imagined as hexagonally shaped, with portal triads forming the corners and central vein in the middle, overlying the bile canaliculi and sinusoidal epithelial network (Burt, 2011).

Repetition of a toxic insult to the liver is a convenient and easily reproducible way of inducing fibrosis in rodents in the lab (Weiler-Normann, 2007). One of the well-studied hepatotoxins is carbon tetrachloride (CCl_4). CCl_4, a halogenated alkane, is activated by cytochrome CYP2E1 in hepatocytes to form the trichloromethyl radical CCl_3*. This

radical binds to cellular components (nucleic acid, protein, and lipid) and affects crucial processes in the cell, including oxidative stress, lipid metabolism, membrane permeability, and protein synthesis. The injury signal from hepatocytes attracts inflammatory cells, which together activate fibroblasts in the liver to proliferate, synthesize, and deposit ECM proteins along fibrotic septa. Changes in gene expression patterns involved in cell death, proliferation, metabolism, and fibrogenesis match the histological phenotype of necrosis, inflammation, and fibrosis (Jiang, 2004). Since CYP2E1 expression outside the liver is almost non-existent, extra-hepatic damage is minimal.

When given repeatedly at low doses, all of the CCl_3* radical effects occur in parallel and successive rounds of wound healing occur prior to the resolution of the previous one, resulting in fibrosis (Starkel and Leclercq, 2011). Intra-peritoneal injection of low doses of CCl_4 for up to 3 months results in pericentral fibrosis, with both centro-central (linking two central veins) and centro-portal (linking a central vein and a portal triad) septa (band). This feature, with deposition of ECM along septa that extend along liver lobules with connected portal tracts and central veins in various arrangements, is called bridging fibrosis. Central-portal bridging fibrosis is the most distinct histologic phenomenon seen in clinical patients with chronic active hepatitis (Lee, 1994). Thus, CCl_4 injection mimics phenotypes observed in the clinic and can be a method for understanding pathological mechanisms of liver fibrosis.

No current anti-fibrotic treatment exists; new approaches include targeting inflammatory or signaling proteins and miRNAs. One of the well-established miRNAs in liver fibrosis is the miR-29 family members. The miR-29 family consists of miR-29a,

miR-29b, and miR-29c, in two different genomic locations. Human miR-29a and miR-29b1 as well as miR-29c and miR-29b2, are encoded and co- transcribed as two genes located on chromosome 7 or chromosome 1, respectively. All three miRNAs share the same seed sequence at nucleotides 1 to 8. miR-29 family target many ECM members including collagens, elastin and fibrillin (Li, 2009; Ott, 2011; van Rooij, 2008). Downregulation of miR-29 family has been reported in cardiac (van Rooij, 2008), kidney (Wang, 2012), lung (Cushing, 2011), liver (Kogure, 2012) fibrosis and systemic sclerosis (Maurer, 2010). This downregulation has also been reported in clinical patients with liver fibrosis (Roderburg, 2011).

Therapeutic approaches to normalize the expression of miRNAs has been studied using various delivery systems. MiRNAs make great therapeutic agents in-vivo because of their small size, relative stability, diverse target recognition, and lack of adverse effects observed so far (Kota, 2009). Adenoviral delivery of let-7 microRNA has led to regression of lung tumor in mice (Trang, 2010). Double stranded miRNA mimics or single stranded antisense nucleotides antagomirs make safe and effective delivery possible and are promising strategies for many cancers (Thorsen, 2012). For instance, miR-10b antagomir inhibited metastasis in a breast cancer mouse model (Ma, 2010) and tail-vein injections of miR-16 mimic complexed with atelocollagen (a low immunogenic form of collagen type 1) inhibited growth and metastasis of prostate tumors in bone (Takeshita, 2010).

In this chapter, I describe the induction of fibrosis in mice using CCl_4 as the insult and documented miR-29a family down regulation during the progression of fibrosis. We adopted an adenoviral delivery system described before (Kota, 2009) to overexpress miR-

29a in mice livers before and after histologically established fibrosis. Viral expression was observed only in hepatocytes in the liver but was still able to successfully prevent and block the progression to a fibrotic phenotype. We demonstrate that a novel delivery system to sustain the expression of miR-29a in the parenchymal compartment of fibrotic mouse livers was successful in preventing and completely reversing fibrosis.

2.2 Results

2.2.1 miR-29 is downregulated in fibrotic mouse livers

Liver fibrosis was induced in mice livers by injection of CCl_4 intra-peritoneally twice a week for up to 8 weeks and progressively increasing fibrotic phenotype was observed (Figure 2-1a). We profiled miRNA expression changes over time to identify miRNAs possibly modulating fibrosis. 518 unique mouse miRNAs were assayed on an array and many showed dysregulation of expression (Figure 2-1b). Interestingly, all three members of the miR-29a family showed down regulation over time and severity of fibrosis, which was validated by individual taqman assay. (Figure 2-1c). Analysis of the primary transcript of miR-29a/b1 showed no change under the same conditions, indicating a post-transcriptional mechanism of decrease in miR-29a (Figure 2-1c).

TGF-β has been shown to be one of the factors responsible for the decrease in mature miR-29 expression (Van Rooij, 2008). TGF-β mediated down regulation of miR-29 has also been shown in the immortalized stellate cell line LX2 (Bandopadhyay, 2011). We treated human hepatocytes with TGF-β in-vitro and observed similar down regulation of all three members of miR-29 family (Figure 2-1d), indicating that the down regulation observed in whole liver tissue from fibrotic mice could be a reflection of the down

regulation of miR-29 in both myofibroblast and hepatocyte compartments. As seen in whole liver, this decrease was also post-transcriptional, demonstrating no change in primary miR-29a/b1 expression (Figure 2-1d).

2.2.2 Systemic delivery of miR-29a into fibrotic livers showed hepatocyte specific expression

Adenovirus-mediated delivery of miR-29 has previously been shown to attenuate CCl_4-induced liver fibrosis (Zhang, 2012). We tested a more clinically relevant delivery system employing scAAV (self-complementary adeno-associated virus) with an AAV8 liver-specific serotype (Kota, 2009). This vector has enhanced GFP (eGFP) under the control of a ubiquitous promoter elongation factor 1 alpha (EF1α). We created scAAV.miR-29a.eGFP by cloning miR29a into the short intron that is contained in the EF1a promoter unit, facilitating the simultaneous production of miR-29a and eGFP from a single transcript (Figure 2-2a). Expression of the plasmid was confirmed by transfecting into HEK293 cells and corresponding increase in mature miR-29 expression with increasing plasmid concentration was observed (Figure 2-3a)

2×10^{11} vector genomes (vg) of scAAV.eGFP (control) or scAAV.miR29a.eGFP were administered to animals via a single tail-vein injection and assessed after 4 weeks for toxicity and vector expression. No gross architectural or pathological changes were observed (Figure 2-2b). Viral genomic DNA and transgene (GFP) expression was detected in DNA and mRNA respectively (Figure 2-2c). At this dose, increased expression of mature miR-29a or primary miR-29a/b1 was not observed (Figure 2-2d). Increasing the dose of scAAV.miR29a.eGFP virus fivefold to 1×10^{12} vector genomes

(vg) did not increase mature miR-29a expression (Figure 2-3b) even though the same viral load increased miR-26a expression in the same vector system in liver (Kota, 2009). Approximately 50% of hepatocytes were transduced and resident stellate cells did not show any GFP expression by desmin/vimentin marker staining (Figure 2-2e-i). Even though the viral genome count and GFP/miR-29a transcript level increases, there is no corresponding increase in mature miR-29a expression. The independence of viral load in contributing to miR-29a expression indicates that there is tight regulation of miR-29 maturation in-vivo.

2.2.3 Pretreatment with miR-29a-AAV sustains expression of miR-29a and prevents fibrosis

Since mir-29a is down regulated in hepatic fibrosis, we wanted to test the ability of the scAAV.miR29a.eGFP in modulating fibrotic phenotype. Mice were given either 2×10^{11} vg of scAAV.eGFP or scAAV.miR29a.eGFP in a single tail-vein injection 1 week before 4 weeks of CCl_4 mediated liver injury (Figure 2-4a). After 4 weeks of CCl_4, scAAV8.eGFP treated mice showed reduced miR-29a expression and significant increases in histological and biochemical measures of fibrosis (Figure 2-4c, d). In contrast, scAAV8.miR29a.eGFP treated mice exhibited normal (equivalent to endogenous) hepatic miR-29 expression (Figure 2-4c), and lacked any histologic evidence of fibrosis (Figure 2.4d) and exhibited a slight increase in total collagen (Figure 2-4f). At the end of 4 week analysis, viral load was dramatically decreased in control virus treated fibrotic animals while the scAAV8.miR29a.eGFP treated group had a slighter decrease in viral genome copy number (Figure 2-4b). Blinded scoring of fibrosis

showed dramatically lowered scores for miR-29a virus treated animals compared to control virus group (Figure 2-4e).

2.2.4 miR-29a-AAV reverses fibrotic phenotype in established and ongoing injury

We established that miR-29a-AAV could protect against fibrosis in pre-treated mice and wanted to test if the virus could also block or even reverse in the context of well-established liver injury. To determine this, a single dose of 2×10^{11} vg of scAAV.eGFP or scAAV.miR29a.eGFP was injected into tail-vein of mice on week 5 of a 12 week course of CCl_4 treatment (Figure 2-5a). Liver injury and associated hepatocyte proliferation has been known to rapidly dilute AAV vector genomes (Song, 2004) and 8 weeks after injection (12 weeks total CCl_4) viral genomes were very low in both control and miR-29 treated animals (Figure 2-5b). Accordingly, miR-29a expression was also significantly reduced in both scAAV8.eGFP and scAAV8.miR29a.eGFP treatment groups (Figure 2-5c). Nevertheless, mice that received scAAV8.miR29a.eGFP had significantly lower fibrosis than scAAV8.eGFP-treated mice (Figure 2-5e) and much reduced collagen protein expression (Figure 2-5f). Further, blinded histopathologic scoring revealed that miR-29a-treated mice had less evidence of fibrosis than was present at the time of AAV injection (Figure 2-5e), indicating that therapeutic delivery of miR-29a resulted in reversal of established fibrosis despite the continued administration of CCl_4.

2.3 Discussion

Currently, no approved therapies exist for treating fibrosis or to target the core pathway of ECM synthesis and deposition. miR-29 directly suppresses many proteins in the ECM (including collagens) and is down regulated in fibrosis, indicating that it plays an important role in progression of fibrosis. Indeed, the anti-fibrotic effects of miR-29a have been shown in liver (Zhang, 2008), lung (Xiao, 2012), heart (van Rooij, 2008), and muscle (Wang, 2012).

miR 29a/b1 transcript is up regulated by hepatocyte growth factor (Kwiecinski, 2011), and estradiol (Zhang, 2012) and transcriptionally down regulated by TGF-β through AP-1(Huang, 2012). TGF-β also repressed miR-29b2/c through smad3 (Qin, 2011). In our CCl_4 treatment in mice, and extraneous addition of TGF-β to human hepatocytes, we observed novel post-transcriptional decrease of miR-29a. Post-transcriptional down regulation of miRNA can be regulated at multiple levels- by regulating Drosha, export from the nucleus, Dicer, argonaute binding or additionally, other factors which affect cleavage by drosha/dicer (Ma and Huang, 2010). The post-transcriptional mechanism of down regulation of miR-29a is currently unknown but could still prove to be a useful clue in understanding how initial injury response(s) in fibrosis control mature miR-29a expression.

We showed that restoration of expression of miR-29 using scAAV vector was successful in preventing and reversing fibrosis. Single stranded self-complementary AAV vectors can spontaneously re-anneal and have no need of host cell DNA synthesis. This greatly increases their efficiency of transduction (Mccarty, 2001) and their ability to infect both dividing and non-dividing cells. These vectors have successfully been used in primates and even in the clinic e.g. for hemophilia (Nathwani, 2007). Till today, no

pathogenicity has been observed for scAAV and they are in use in several clinical trials (Mingozzi and High, 2011).

We and another group (Zhang, 2012) have showed that overexpressing miR-29a before the onset of fibrosis is protective and demonstrated the anti-fibrotic potential of miR-29a. We are the only group to have also proved the efficacy of the protective role of miR-29a in a clinically relevant setting, after progressive fibrosis is established. We have added onto existing data, verifying anti-fibrotic effects of miR-29a, and paved the way for clinical approaches to effectively treat fibrosis.

In 2009, Kota *et al*., reported that mice transduced with $1x10^{12}$ scAAV8.miR26a.eGFP genomes exhibited significant overexpression of miR-26a in liver with transduction of over 90% of hepatocytes. In contrast, we could not find evidence of increased miR-29a expression in the livers of uninjured mice that received a comparable dose of scAAV8.miR29a.eGFP (Figure 2-3b). This observation suggests that maturation of miR-29a is tightly regulated under normal physiologic conditions and therefore the addition of virally produced precursor transcripts does not result in a net increase in mature miR-29a levels. Nevertheless, we demonstrated that a much lower dose of $2x10^{11}$ vg was sufficient to restore hepatic miR-29a expression in CCl_4 treated mice, indicating that the setting of chronic liver injury is permissive for processing of the virally-derived miRNA transcript. This regulatory mechanism may also provide natural protection against potential toxicity from virally-derived miR-29 overexpression.

In the liver, the resident fibroblasts majorly contributing to the myofibroblast population are called hepatic stellate cells. Myofibroblasts are the most common source of collagen and other ECM proteins. Many reports have suggested that the decrease of

miR-29 in fibroblasts is mediated by the profibrotic cytokine TGF-β (Roderburg, 2010) and here, we showed that miR-29 is also well expressed in hepatocytes, and is down regulated by TGF-β as well. Our data indicates that restoring the expression of miR-29 in hepatocytes alone is sufficient to rescue mice from a fibrotic phenotype. AAV8 has been shown to efficiently transfect hepatocytes (Nathwani, 2007; and Kota, 2009) and we observed about 50% transduction of hepatocytes with our viral load ($2x10^{11}$ vg). No co-localization of GFP was observed with stellate cell markers desmin and vimentin, indicating that our virus did not transfect fibroblastic cells. Still, fibrosis was effectively blocked and even reversed in CCl_4 injured mice. There are several possible explanations which could explain the above observation-

- miR-29 targets upstream pro-fibrotic pathways in hepatocytes and/or stops the progression of injury signal transfer to stellate cells.
- miR-29 functions in a paracrine fashion and is transferred from hepatocytes to stellate cells through endosomes

There is some evidence to support both of these explanations- miR-29 targets many proteins that are upstream players in fibrosis which could be expressed in hepatocytes as well (Table 2.1) and there are reports of transfer of miRNA to neighboring cells through exosomes (Valadi, 2007; Katokowski, 2010; (Mittelbrunn, 2011; and Montecalvo, 2012). Our observations are consistent with a prior report that hepatocyte specific knockout of miR-29 was associated with increased collagen deposition in a mouse model of liver fibrosis (Kogure, 2012).

We also assessed the durability of our delivery model by counting viral genomes and assessing GFP positivity and miR-29a expression. Since AAV are replication

defective and extensive injury to hepatocytes occur during fibrosis (Wallace K, 2008), we observed substantial reduction in viral genomes. In the pre-treatment model, we observed a much more severe loss of viral genomes in the control virus treated group compared to the scAAV.miR29a.eGFP virus treated animals. The partial preservation of viral genomes supported the evidence that the miR-29a virus group sustained mature miR-29a expression but was lost in the control virus group, which was sufficient to block the progression of fibrosis. This suggests the miR-29a not only prevents ECM production (fibrosis) but also hepatocyte death/proliferation, since this is the main mechanism of loss of virus.

In the instance of ongoing injury, when the miR-29a virus was injected during the course of CCl_4 treatment, we observed almost complete loss of viral genomes in both groups at the end of the course, 12 weeks. Correspondingly, at this time point, mature miR-29a expression was the same irrespective of the virus received and was equivalent to the down regulation seen with CCl_4 injury. Interestingly, a significant percentage of hepatocytes were still GFP+ suggesting that AAV genomes were only recently lost and thus virally-mediated miR-29a expression was present for most of the 8 week period. This suggests that if we our end point had moved up by a week, we would have seen a definite presence of viral genomes and mature miR-29a expression, explaining the lack of fibrosis observed in the miR-29a virus group of animals.

2.4 Conclusions

To summarize, in this chapter we confirmed the down regulation of miR-29a in a CCl_4 mediated model of liver fibrosis. Our data demonstrates that a single injection of

scAAV8.miR29a.eGFP ameliorates fibrosis when administered prior to or after the onset of liver fibrosis, and highlights the importance of sustaining miR-29a expression during fibrosis. Since ECM synthesis/deposition is the main downstream process in all forms of fibrosis and the down regulation of miR-29 has been observed in multiple organs and different injury mediated fibrosis, restoration of miR-29a expression may be beneficial in treating a variety of fibro proliferative disorders. This form of gene therapy using scAAV is being used to treat hemophilia B (Nathwani, 2011) and miR-122 is the first miRNA to be in second stage clinical trials to treat HCV infection (Jannsen, 2013). Indeed, miR-29a is one of the miRNAs in the pipeline at miRagen Therapeutics, Inc., for treatment of pathological fibrosis, illustrating the potential for clinical success.

2.5 Materials and methods

2.5.1 Carbon tetrachloride (CCl_4) treatment and vector delivery, statistics

C57/BL6 mice received intraperitoneal injections of 1 ml/kg carbon tetrachloride (Sigma-Aldrich) diluted 1:7 in corn oil twice a week for up to 12 weeks. scAAV.miR29a.eGFP was constructed by amplifying miR-29a from human genomic DNA using the following primers: 5'-ATACCGGGCCGGCCGAGCCCAATGTATGCTGGAT-3' (forward) and 5'-ATACCGGGCCGGCCTGCATTATTGCTTTGCATTTG-3' (reverse). The amplicon was cloned into the FseI site of scAAV.eGFP (Kota J., *et al*., 2009). AAV was administered at a dose of $2x10^{11}$ viral genomes (vg) per animal (all Figures) or $1x10^{12}$ vg/animal (high dose; Figure 2 only) via tail vein injection with a 30g needle. The Johns

Hopkins School of Medicine Animal Care and Use Committee approved all housing and procedures.

5 μm sections of formalin fixed, paraffin embedded liver samples were cut and stained with hematoxylin and eosin as well as Masson's trichrome by the Johns Hopkins Reference Histology Lab (Baltimore, MD). A trained pathologist, who was blinded to AAV and CCl_4 treatment details, scored the hepatic fibrosis of each animal on a scale of 0-4.

All statistical comparisons of qPCR data were performed using REST 2009 software (Qiagen). A two-tailed T-test was used to calculate p values for comparisons of fibrosis scores and quantitative collagen assays.

2.5.2 MicroRNA array

Total liver RNA was prepared using a mirVana miRNA Isolation Kit (Ambion, Inc, Austin, TX) according to the manufacturer's protocol. 500ng RNA was reverse transcribed without pre-amplification using Megaplex RT Primers (Rodent Pool A v2.0; Applied Biosystems) and a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's protocol. The microarray was run on the 7900HT Fast Real-Time PCR System with TaqMan Array Rodent MicroRNA A Cards v2.0 (Applied Biosystems), according to the manufacturer's protocol. miRNA expression levels after 1, 4 and 8 weeks of CCl_4 exposure (compared to normal liver) were plotted as a heatmap using the statistical programming language R. The geometric mean of each array plate was used for normalization and the top 70 miRNAs with the largest average fold change were sorted by fold change at 8 weeks.

2.5.3 Immunostaining and collagen assay

Formalin-fixed, paraffin-embedded tissues were sectioned. 5 μm sections were transferred to Superfrost/Plus Microscope Slides (Fisher Scientific) and melted onto the slide for 30' at 60°C. After washing in PBST, samples were blocked with PBS + 5% fetal bovine serum (Sigma) and 3% goat serum (Sigma). Slides were then incubated for 1 hour at room temperature with antibodies specific for albumin (Santa Cruz Biotechnologies, Inc), vimentin (Millipore, Billerica, MA), desmin (Sigma), or GFP (Invitrogen). They were then washed 3 times in PBST and incubated for one hour at room temperature with combinations of the following secondary antibodies: Cy3 labeled goat anti-rabbit IgG (GE Healthcare, Niskayuna, NY), Cy3 labeled anti mouse, Cy3 anti-chicken, (both Millipore), or Alexa Fluor 488 anti-rabbit (Cell Signaling Technology, Danvers, MA). Slides were washed three times in PBST, counterstained with Hoechst 33258 (Molecular Probes, Eugene, OR), and mounted using Prolong Gold Antifade Reagent (Invitrogen). Collagen levels were determined by Sircol Soluble Collagen Assay (Biocolor), which was performed according to the manufacturer's protocol.

2.5.4 RNA, DNA isolation and PCR

Total RNA was isolated from cultured cells or whole liver tissue using Trizol (Invitrogen) and treated with DNAse (DNAse I Amplification Grade; Invitrogen) according to the manufacturers' protocols. Expression of 18s rRNA, primary miR-29a, mature miR-29a-c, miR-122 and miR-130a was assessed using individual Taqman assays (Applied Biosystems). Non-quantitative amplification of viral gDNA and cDNA was performed using DreamTaq Green Master Mix (Fermentas) according to manufacturer's

protocol using the following primers: 5'-CGCAACGGGTTTGCCGCCAGAAC-3' (forward); 5'-GGCCGTTTACGTCGCCGTCCAG-3' (reverse).

A portion of the viral genome (5'-CCACTACCTGAGCACCCAGTC-3' (forward); 5'-TCCAGCAGGACCATGTGATC-3' (reverse)) and a non-repetitive locus in the mouse genome (DGCR8;

5'-CCATCAGGCAATGGCTCTGT-3' (forward);

5'TGCAGGATGTTTTTTGTGTTCTG-3' (reverse)) were separately amplified from whole liver genomic DNA samples from transduced mice. Standard curves of known amounts of AAV8.eGFP plasmid DNA ($2.96x10^{-6}$ pg per copy) and whole liver genomic DNA (5.8 pg dsDNA per diploid cellular genome) were used to determine the number of viral and cellular genomes in each sample.

2.5.5 Addition of TGF-β to hepatocytes in-vitro

Human hepatocytes (CellzDirect, Durham, NC) were plated on dishes coated with 5μg/ml collagen (Gibco, Grand Island, NY) in serum-rich media (DMEM +10% FBS, 15mM Hepes, 10μg/ml gentamycin (Quality Biological, Gaithersburg, MD), 1x ITS (Sigma), 1mM dexamethasone (Sigma), and 2mM L-glutamine (Quality Biological)). 24h later, the cells were washed and the media was replaced with serum-free media. After 24h of serum starvation, the cells were again washed and fresh serum-free media with or without 5ng/ml TGF-β (Roche) was added. RNA was isolated 24 hours after the addition of TGF-β.

2.6 Tables: Chapter 2

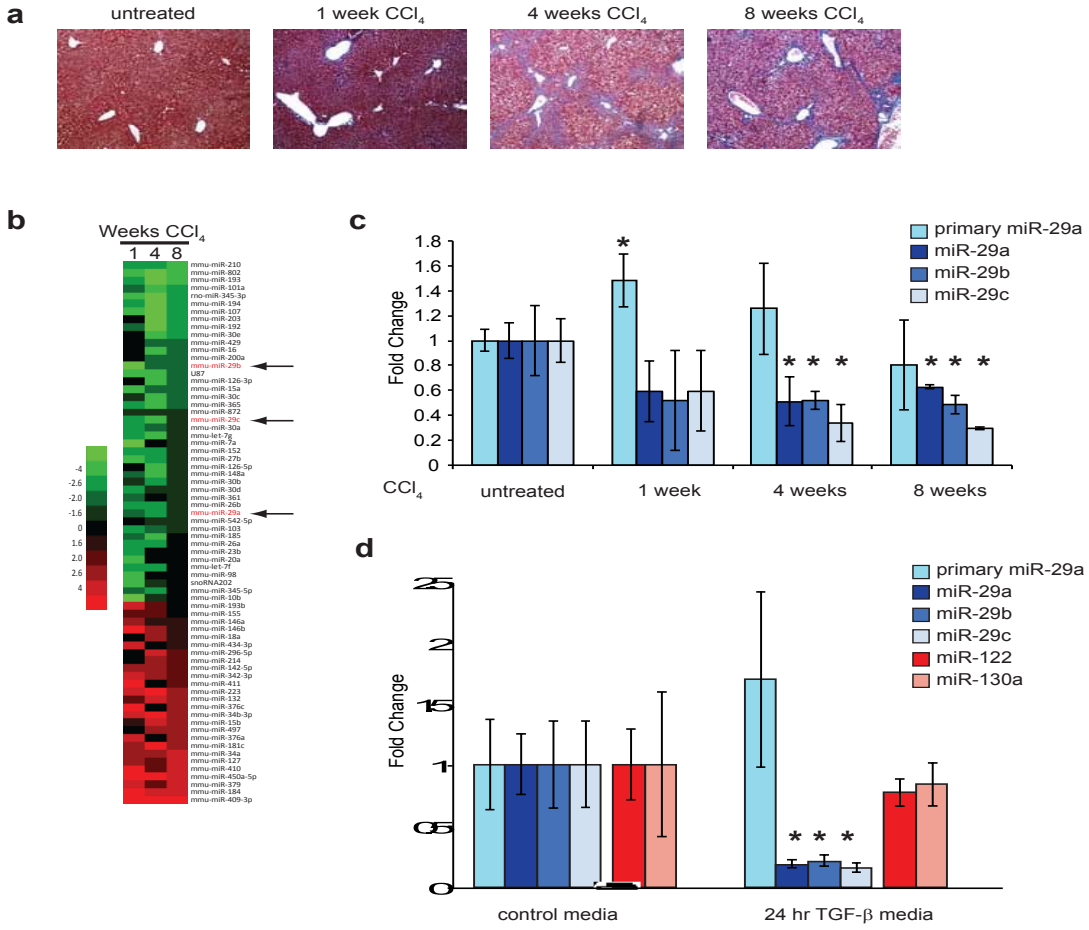
Table 2-1. Examples of non-ECM targets of miR-29 implicated in fibrosis

		PMID	
	name of gene	Fibrosis	Target of miR29
1	BAX	24052410	
2	BCL-2	24052410	
3	HBP1	22646479	
4	DICER1	18809798	21761362
5	DNMT3B	24138392	24138392
6	PTEN	24138392	23143395
7	LEPTIN	23295202	
8	IFN- g	24136786	21785411
9	IGF-1	23987664	23183171
10	PDGF-C	22565577	22565577

Pubmed IDs for publications for confirmed targeting by miR-29 and involvement in fibrosis is given for each predicted target of miR-29.

2.7 Figures: Chapter 2

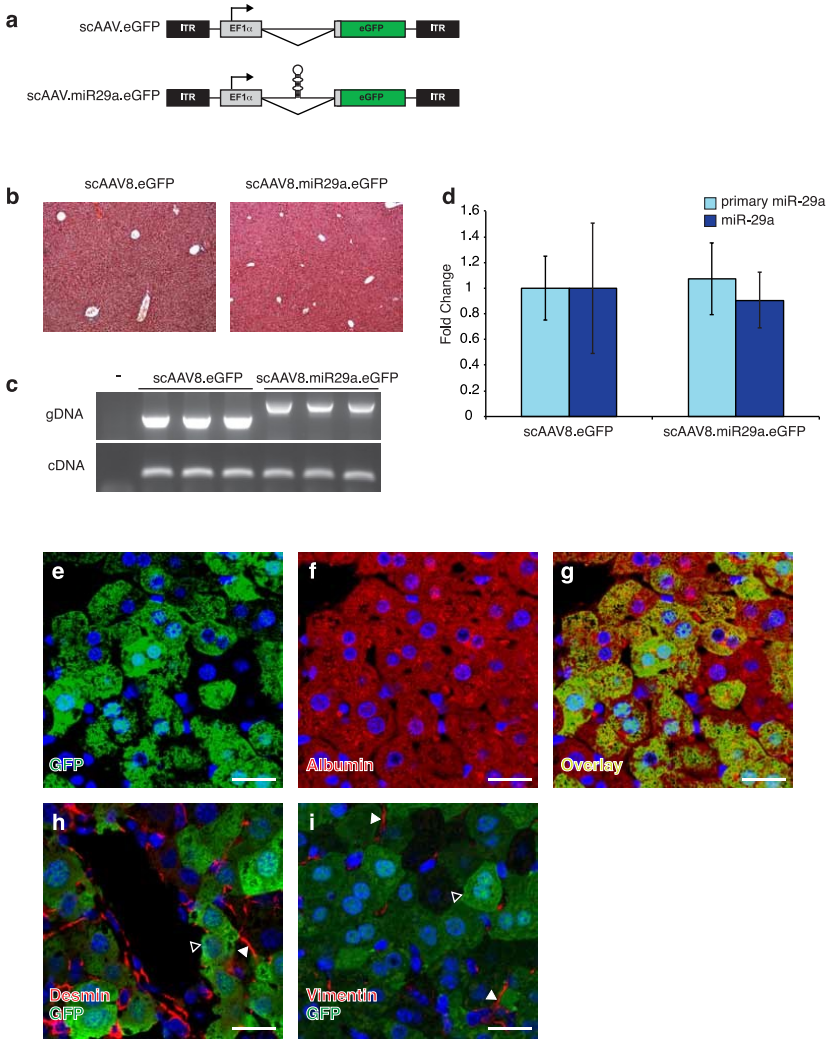
Figure 2-1. Primary and mature miR-29 expression in murine liver and isolated hepatocytes



(**a**) Carbon tetrachloride-mediated liver fibrosis. Trichrome-stained liver sections demonstrating progressive fibrosis during 8 weeks of CCl_4 exposure. (**b**) Heat map of miRNA expression levels after 1, 4 and 8 weeks of CCl_4 exposure (compared to normal liver). The top 70 miRNAs with the largest average fold change are shown and sorted by fold change at 8 weeks. miR-29 family members indicated with arrows. (**c**) Hepatic expression levels of primary and mature miR-29a,b,c in CCl_4-treated mice. Average fold changes for 1 week (n=4), 4 week (n=3) and 8 week (n=2) treatment groups were calculated using normal liver (no CCl_4) as a reference (n=2). Error bars represent +/- one standard deviation. (*= $p<0.05$ compared to normal liver). (**d**) TGF-β

represses miR-29 expression in human hepatocytes. Average fold change of primary and mature miR-29a,b,c as well as miR-122 and miR-130a in TGF-β treated hepatocytes were calculated using control media-treated cells as a reference. Error bars represent +/- one standard deviation. (*= $p<0.05$ compared to control media).

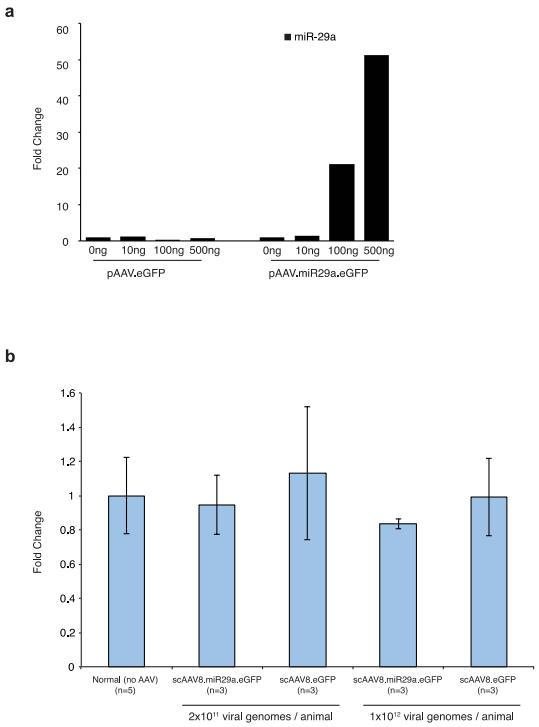
Figure 2-2. scAAV8 transduction and miR-29 expression levels in murine liver.



(**a**) Schematic representation of scAAV vectors depicting locations of inverted terminal repeats (ITRs), elongation factor 1-alpha promoter (EF1α), miRNA (shown in hairpin form), and enhanced green fluorescent protein (eGFP) open reading frame. (**b**) Transduction with scAAV8 does not disrupt normal liver architecture. Trichrome stained liver sections from AAV-transduced animals demonstrating normal histology. (**c**) Viral genomic DNA (gDNA) and mRNA from the EF1α transcription unit (cDNA) are readily detectable in mouse liver following

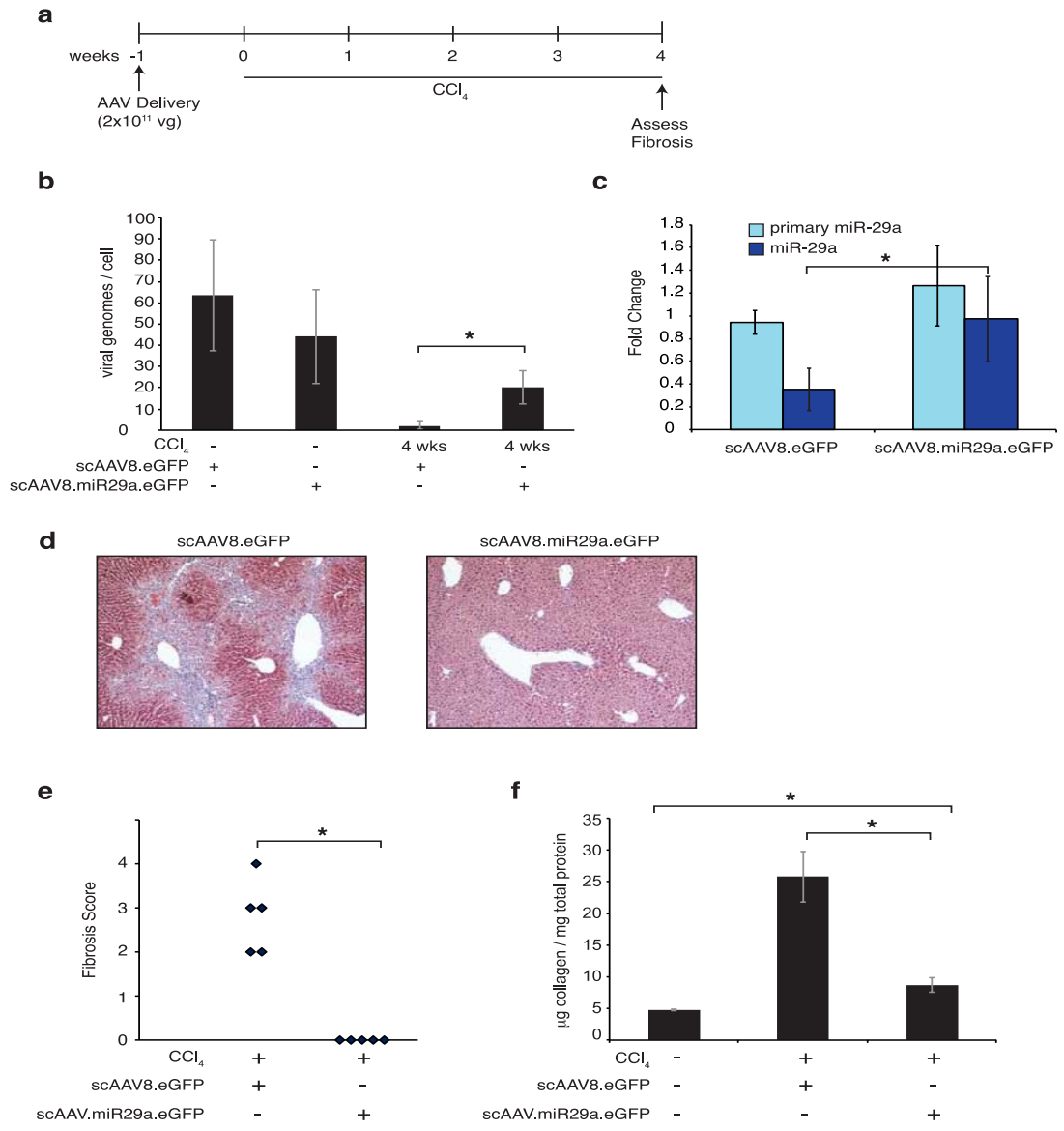
transduction with $2x10^{11}$ vg of scAAV8.eGFP (n=3) or scAAV8.miR29a.eGFP (n=3). The presence of the hairpin accounts for the increased size of the scAAV8.miR29a.eGFP gDNA amplicon. (**d**) Hepatic expression of primary and mature miR-29a in scAAV8 transduced mice. Average fold change for each treatment group was calculated using scAAV8.eGFP treated mice as a reference (n=3). Error bars represent +/- one standard deviation. (**e-i**) Localization of AAV-mediated GFP expression in transduced mouse liver. Sections of transduced livers were co-immunostained for GFP (**e,g,h,i**; green) and markers of hepatocytes (Albumin **f,g**; red) or stellate cells (Desmin **h**; Vimentin **i**; red). Open arrowheads indicate GFP+ hepatocyte and filled arrowheads indicate desmin+ or vimentin+ stellate cells. All sections were counterstained with Hoechst (blue). Confocal images were captured with a 40x objective and are shown at 2X zoom. Scale bar = 20μm

Figure 2-3. In vitro and in vivo miR-29 expression levels associated with AAV.miR-29.eGFP.



(a) miR-29a expression in HEK293 cells transfected with varying amounts of AAV.eGFP or AAV.miR-29a.eGFP plasmids. Average fold change was calculated using mock transfected (0 ng) cells as a control. **(b)** Hepatic mir-29a expression in mice receiving a single injection of low dose ($2x10^{11}$ vg) or high dose ($1x10^{12}$ vg) AAV. Average fold change was calculated using normal (no AAV) as a control. Error bars represent +/- one standard deviation.

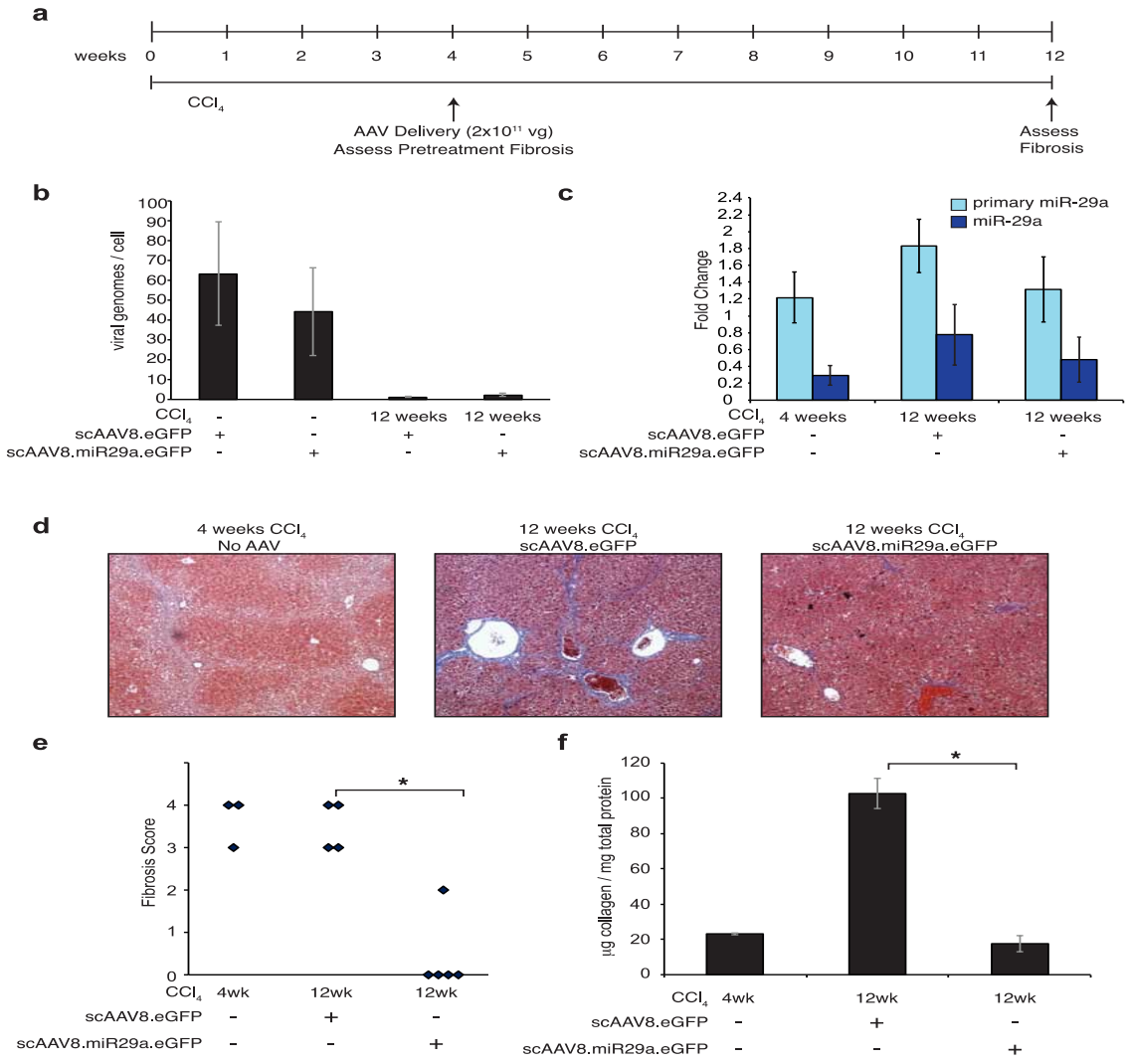
***Figure 2-4.* Pre-treatment with scAAV8.miR29a.eGFP prevents CCl_4-mediated hepatic fibrosis.**



(**a**) Timeline of AAV delivery and CCl_4 treatment. (**b**) Estimate of viral genomes/cell in livers of scAAV-transduced mice. A portion of the viral genome (GFP) and a non-repetitive locus in the mouse genome (DGCR8) were separately amplified from whole liver genomic DNA. Standard

curves of known amounts of AAV8.eGFP plasmid DNA and whole liver genomic DNA were used to determine the number of viral and cellular genomes in each sample, respectively. (**c**) Hepatic expression of primary and mature miR-29a in the livers of CCl_4-treated scAAV8.eGFP (n=5) and scAAV8.miR29a.eGFP transduced animals (n=5). Average fold change for each treatment group was calculated using normal (no CCl_4) scAAV8.eGFP treated mice as a reference (n=3). Error bars represent +/- one standard deviation. (**d**) Trichrome staining reveals reduced collagen deposition (blue) in scAAV8.miR29a.eGFP transduced livers. (**e**) The degree of fibrosis was scored on a scale of 0-4 by a trained pathologist (blinded to treatment condition) and the score for each individual animal is shown. (**f**) Quantitative determination of hepatic collagen levels in transduced animals. Error bars represent +/- one standard deviation. (*= $p<0.05$).

Figure 2-5. Intervention with scAAV8.miR29a.eGFP reverses histologic evidence of CCl_4-mediated hepatic fibrosis



(**a**) Timeline of AAV delivery and CCl_4 treatment. (**b**) Estimate of viral genomes/cell in livers of scAAV-transduced mice. A portion of the viral genome (GFP) and a non-repetitive locus in the mouse genome (DGCR8) were separately amplified from whole liver genomic DNA. Standard curves of known amounts of AAV8.eGFP plasmid DNA and whole liver genomic DNA were used to determine the number of viral and cellular genomes in each sample, respectively. (**c**) Hepatic expression of primary and mature miR-29a in CCl_4-treated scAAV8.eGFP (n=4; one of five scAAV8 injected mice died during CCl_4 treatment) and scAAV8.miR29a.eGFP transduced animals (n=5). Three animals were sacrificed after 4 weeks of CCl_4 treatment to establish the level of fibrosis present at the time of viral delivery (pre-treatment). Average fold change for each

treatment group was calculated using normal (no CCl_4) scAAV8.eGFP treated mice as a reference (n=3). Error bars represent +/- one standard deviation. (**d**) Trichrome staining reveals reduced collagen deposition (blue) in scAAV8.miR29a.eGFP transduced livers compared to either pre-treatment (4 weeks CCl_4) or scAAV8.eGFP treatment (12 weeks CCl_4). (**e**) The degree of fibrosis was scored on a scale of 0-4 by a trained pathologist (blinded to treatment condition) and the score for each individual animal is shown. (**f**) Quantitative determination of hepatic collagen levels in transduced animals. Error bars represent +/- one standard deviation. (*= $p<0.05$)

3. CHAPTER 3. miR-18a IS INCREASED IN ALCOHOLIC LIVER FIBROSIS AND REGULATES TGF-β SIGNALING

3.1 Introduction

Alcohol abuse in developed countries is widespread and has various short-term and long-term health risks. Approximately 80,000 deaths are attributable to excessive alcohol use per year in the United States, with 15% of these specifically due to alcoholic liver disease (CDC report, 2001-05). Long term alcohol use damages the brain, heart, intestines, liver and is also a risk factor for certain cancers (CDC datasheet). In the liver, there are three types of alcohol-induced liver disease- fatty liver, alcoholic hepatitis, and cirrhosis. Cirrhosis is defined as end-stage, irreversible fibrosis of the liver and was the 12th leading cause of death in USA in 2010 (Murphy, 2013). The only therapy for these late-stage patients is liver transplantation, which is heavily burdened by lack of donor organs and long waiting times. Approximately 2500 people died while on the waiting list in 2011 (Annual Report, HHS). This makes alcohol mediated cirrhosis one of the most expensive clinical burdens of our times and no current therapies exist to prevent or delay the progress to cirrhosis.

Alcohol mediated effects in the liver include hepatitis (inflammation of the liver), steatosis (fat accumulation), and fibrosis. Ethanol is processed by hepatocytes and is broken down into two pro-fibrotic agents- acetaldehyde and reactive oxygen species (ROS). These, diffuse outside to hepatic stellate cells (HSC), leading to up regulation of inflammatory mediators and cytokines such as TGF-β (Purohit and Brenner, 2006). A pro-fibrotic cascade of changes is activated, resulting in increased synthesis and

deposition of ECM by activated myofibroblasts, ending in destruction of liver architecture, and parenchyma replaced by non-functioning scar tissue. This leads to functional consequences, including symptoms of jaundice and hypertrophied liver from the hepatitis, and portal hypertension and ascites from cirrhosis, ultimately leading to hepatocellular carcinoma.

In human patients, typically alcohol use occurs for decades with varying intakes, which ultimately results in cirrhosis. Replicating the same in animal models is often difficult because, fibrotic symptoms are seldom observed in ethanol feeding alone, unless coupled with a secondary "hit" such as dietary changes or hepatotoxin usage (Arteel, 2010). This has led to the rise of a "second hit" model of alcoholic liver disease, with interplay of ethanol abuse and other environmental insults (such as drugs, hepatitis viruses etc.) (Tsukamoto and Lu, 2001). Direct ethanol feeding to rodents through a liquid diet has been employed to mimic alcohol abuse in patients and this model mimics the early phenotype of steatosis (fat droplets accumulation in liver, confirmed by histopathology). The ethanol fed animals do not progress to a severe fibrotic/cirrhotic phenotype even after prolonged exposure to ethanol, indicating that there are additional risk factors necessary for cirrhosis (Lieber and DeCarli, 1989). As indicated in chapter 2, CCl_4 exposure for a month can be severely fibrotic. Models using both ethanol and CCl_4 have been reported to effectively induce fibrosis in the liver (Takeuchi, 1968; Plummer, 1994). Ethanol induces stabilization of CYP2E1 (Roberts, 1995) - the main cytochrome involved in processing CCl_4, thus potentiating the effects of CCl_4 toxicity and shortening the time of onset of fibrosis. We sought to use a model with ethanol and a low dose of

CCl_4 to mimic the symptoms of necrosis, steatosis and fibrosis as seen in patients in the clinic.

As in almost any form of fibrosis, TGF-β is the main pro-fibrotic pathway mediator in alcoholic fibrosis (Siegmund, 2005). Many microRNAs target proteins involved in TGF-β signaling (refer Chapter 1.5) and in this chapter, I will focus on the protective roles of miR-18a in alcohol induced fibrosis. miR-18a belongs to a cluster of 6 miRNAs called the miR 17-92 cluster. miR 17-92 cluster has 6 miRNAs – miR-17, miR-18a, miR-19a , miR-20a, miR-19b1, miR-92a1 and was first identified to be up regulated by c-myc (O'Donnell, 2005) and subsequently found to be amplified/overexpressed in many cancers, and is called "oncomir-1" (Olive, 2010). miR 17-92 cluster also has 2 paralogs mir-106a-363 and mir-106b-25, arising from gene duplication in mammals. The three clusters together code for 15 miRNAs, split into 4 groups defined by their shared seed sequences. Together, the 3 clusters have been recognized to be important for B lymphogenesis and lung development (Ventura, 2008), angiogenesis (Dews M, 2010), embryonic palate development (Li, 2012), and in hypoxia induced apoptosis (Yan, 2009).

The three clusters target many proteins involved in TGF-β signaling (Table 3.1); miR-18a specifically targets key proteins in TGF-β signaling, including smad2, smad4, and CTGF, which supports its powerful anti-fibrotic role in fibrosis. In this chapter, I will describe the up regulation of miR-18a in an alcoholic model of fibrosis which suppresses TGF-β signaling and fibrotic effects.

3.2 Results

3.2.1 Global profiling of miRNAs in liver fibrosis reveals miR-18a upregulation

An alcoholic model of fibrosis was setup by combining a Lieber-Decarli liquid diet of ethanol (7%) and a low dose (0.1 ml/kg body weight) of intra-peritoneal injections of CCl_4 twice a week. Three other groups of ethanol only, CCl_4 only and a control diet only were also tested at the end point of 6 weeks. All groups were controlled for the number of calories since ethanol adds more energy to the diet (i.e. isocaloric). Only the combination diet showed a profound portal-portal and centro-portal fibrosis with pseudo lobule formation (Batts and Ludwig, 1995) as indicated by trichrome staining (Figure 3-1a). We also observed steatosis (fat droplet accumulation) and necrosis in the combination group only.

miRNA changes in the four groups were profiled in whole liver RNA to analyze changes in expression in fibrotic and non-fibrotic groups. All groups were normalized to the control diet group and sorted by the combination group. The top 50 most changed miRNAs are plotted in the heat map (Figure 3-1b) and the top most changed miRNA is miR-18a. Baseline expression of miR-18a was not high but it increased significantly in the fibrotic animal livers; verification by taqman (Figure 3-1c) indicated a four-fold increase of miR-18a in the combination treated, fibrotic group. There was some variation within each group, possibly indicating cell autonomous expression in miR-18a or liquid diet induced variability in ethanol consumption (Arteel, 2010).miR-18b which has the same seed sequence as miR-18a but is on a different chromosome was expressed below detection limit in all animals and did not show any change in expression in the array or by individual taqman QPCR.

Analysis of the other miRNAs in the miR 17-92 cluster indicated that two other miRNAs – miR-17 and miR-20a also showed up as upregulated miRNAs in the top 50 most changed heatmap (Table 3.2), in addition to four more miRNAs in the mir-106a-363 and mir-106b-25 clusters. This was suggestive of a transcriptional mechanism at play behind the up regulation of miR-18a. Taqman for mature miR-17 and quantitative sybr-green PCR for pri-miR 17-92 (two different primer sets) demonstrated a non-significant increase in miR-17 and pri miR 17-92 in the combination group only (Figure 3-2a, b). This could not rule out that the increase in miR-18a was transcriptional. (Chaulk, 2011) reported that the miR 17-92 cluster has a compact globular tertiary structure where miRNAs internalized within the core of the folded structure (miR-18a,19b, 92a) are processed less efficiently than miRNAs on the surface (miR-17, 19a, 20) of the structure by drosha. This demonstrates that both transcriptional and post-transcriptional mechanisms control maturation of miRNAs in the miR 17-92 cluster.

3.2.2 hnRNP A1 expression and sub-cellular localization is not different in fibrosis

(Guil and Caceres, 2007; and Ding and Ma, 2009) identified a novel post transcriptional regulation of miR-18a processing by a RNA splicing protein hnRNP A1 (heterogeneous ribonuclear protein A1). Nascent RNA transcripts transcription, splicing, stability, and export through nuclear pores are all mechanisms modulated by this protein (Jean-Phillippe, 2013). hnRNP A1 binds to the hairpin loop and double stranded stem of pri-miR-18a and stabilizes the stem structure, facilitating cleavage by Drosha. hnRNP A1 was shown to be necessary for miR-18a processing in HeLa cells, in-vitro (Guil and

Caceres, 2007). We hypothesized that hnRNP A1 could play a role in the post-transcriptional upregulation of miR-18a in liver.

Whole liver protein lysates were run on a protein gel and probed with anti-hnRNP A1 antibody. No statistically significant differences in hnRNP A1 expression was detected among the four groups, even though there was wide variability of expression within each group (Figure 3-3a). Another possible explanation of hnRNP A1's role in modulating miR-18a expression could be its sub-cellular localization, i.e. hnRNP A1 has to be nuclear to facilitate Drosha processing of miR 17-92 transcript. HnRNP A1 has been reported to move to the cytoplasm in response to osmotic shock (Allemand, 2005) or ER stress (Damiano, 2013). Immunoflourescence of hnRNP A1 revealed widespread expression in the liver with almost 100% nuclear signal in all cells (Figure 3-3b). In all animals, the ratio of nuclear to cytoplasmic signal of hnRNP A1 was always high and no different in fibrotic and non-fibrotic livers, indicating that atleast in our model, hnRNP A1 does not play a role in maturation and up regulation of miR-18a. Further research is necessary to prove the role of hnRNP A1 for post-transcriptional processing of miRNAs in physiological conditions and in-vivo.

3.2.3 In-situ for miR-18a reveals positive fibroblast-like cells only in combination group

Cell specific expression of miRNAs can offer clues to spatial control of their targets expression. I performed an in-situ hybridization of miR-18a on formalin fixed paraffin embedded (FFPE) liver from animals in all the four groups using a DIG labeled LNA (locked nucleic acid) anti-miR-18a oligo probe (Jorgensen, 2010). A scrambled

probe was used as negative control and positive cells were identified by blue stain. All cells were counterstained with nuclear fast red as well.

Bile ducts and hepatocytes were positive in all groups- fibrotic and non-fibrotic (Figure 3-4a). The hepatocyte stained blue in the nucleus, which could be explained by several theories. The probe could bind to unprocessed pri-miR-18a as well, which would be in the nucleus or it could be an artifact arising during processing. Some miRNAs have also been detected in the nucleus (Liang, 2013; Hwang, 2007) and found to regulate other miRNAs and long non-coding RNAs. Since, both the nuclear hepatocyte and bile duct positivity were seen in all the four groups, we did not focus on these cells.

In the combination animal group only, non-parenchymal cells lining the fibrotic septae were positive for miR-18a expression (Figure 3-4a, b). These cells were spindle shaped and were in the collagen producing, trichrome positive region (fibrotic septae). To further confirm the phenotype of these cells, α-SMA (smooth muscle actin) immunofluorescence was performed. α–SMA is one of the markers of activated myofibroblasts (Knittel, 1999), which maybe from many different sources of cells in the liver; including HSCs, resident fibroblasts, bone marrow stem cells, EMT of hepatocytes etc. α–SMA positively stains smooth muscle cells lining the vasculature in normal livers and in control diet, ethanol only and low dose CCl_4 only groups(Figure 3-5a). In the combination animal livers, the same spindle shaped cells (Figure 3-5b) lining fibrotic septae which were miR-18a positive were also α–SMA positive, indicating that the miR-18a positive cells in fibrotic livers are probably fibroblasts. In the liver, HSCs are thought to be the main source of these activated myofibroblasts and the conversion of vitamin A positive dormant stellate cells into collagen producing activated hepatic stellate cells has

been well documented. Induction of α-SMA is the single most reliable marker of stellate cell activation (Friedman, 2008).

3.2.4 TGF-β downregulates miR-18a in-vitro

miR-18a is up-regulated in alcoholic fibrotic liver and its expression is localized to the same region as α–SMA positive cells, lining fibrotic septae around portal-portal and centro-portal connections. LX-2 is a spontaneously immortalized human hepatic stellate cell line which has same physiological profile as stellate cells in-vivo (Xu, 2005). They express key receptors for fibrosis, secrete collagen and have similar gene expression as primary stellate cells. Exogenous TGF-β addition induced elevated pro-fibrotic gene expression (Figure 3-6a) proving additionally the fibrotic nature of LX-2 cells. Uniquely, miR-18a was down-regulated by TGF-β under the same conditions (Figure 3-6b) while pri-miR-17-92 and mature miR-17 did not show any change in expression by Q-PCR (Figure 3-6b). Smad mediated up regulation of miR-21 post-transcriptional processing has been documented (Davis, 2008) but the specific down regulation of miR-18a is novel and the mechanism is unknown. Additionally, miR-18a was also non-significantly down regulated by TGF-β in hepatocytes (Figure 3-6c), indicating that this process is conserved and active in multiple cell types in the liver.

3.2.5 ScAAV8.miR-18a.eGFP did not overexpress mature miR-18a in LX-2

To identify the function of the up-regulated miR-18a in fibrosis, we tested the signaling changes and phenotypes induced by over-expressed miR-18a in LX-2 cells. An AAV-8 based plasmid (as described in chapter 2) was created by inserting miR-18a into

the place of miR-29a to make scAAV8.miR18a.eGFP (Figure 3-7a). Three primer sets of increasing length of miR-18a amplicon lengths, extending to its neighboring miRNAs in the miR 17-92 cluster were used to clone in miR-18a into the AAV8 plasmid (Figure 3-7b). Transfection into LX-2 showed around 40% GFP positive cells suggesting that the GFP-miR-18a transcript was being expressed (Figure 3-7b). When mature miR-18a levels were quantified by Taqman, no increase because of the scAAV8.miR18a.eGFP plasmid was observed (Figure 3-7c). Since scAAV8.miR29a.eGFP plasmid showed elevated miR-29a expression in HEK293 cells (Figure 2-3a), we hypothesized that miR-18a processing is tightly regulated and possibly mediated by secondary structure or regulatory sequences of some distance away from pri-miR-18a, since including the junctions of miR-17a and miR-19a made no difference in miR-18a expression in-vivo.

3.2.6 miR-18a decreased TGF-β mediated signaling and slowed migration of stellate cells in-vitro

We decided to use a double-stranded miR-18a mimic instead to study the effects of up regulating miR-18a in LX-2 cells. Analysis of mature miR-18a level after 24 hours of addition of mimic showed a 1000 fold increase in log scale (Figure 3-8a), a non-physiological expression levels. Still, the exogenous addition of mimic was able to suppress mRNA levels of its targets, CTGF and Smad2 by approximately 50% (Figure 3-8a). Interestingly, no down regulation of another target -Smad4 was observed, indicating that either there is cell specific targets of miR-18a or that our mimic concentration could not reveal the suppression of weaker targets such as Smad4. Smad2 mRNA suppression correlated with its protein down regulation (by ~ 35%) as seen by western blotting (Figure

3.8b). Phosphorylation of Smad2 did not occur in the absence of TGF-β signaling. pSmad3 was expressed at very low levels and was not differently expressed in control and mir-18a mimic transfected LX-2 cells, neither was Smad4 (Figure 3-8b).

Canonical targets of TGF-β such as Smad2 was down regulated by miR-18a and downstream target CTGF was also suppressed but TGF-β has been shown to act through its non-canonical pathways, MAPK, or JNK or p38 kinase in different contexts (Lindsay and Dietz, 2011), resulting in transcription of similar downstream pro-fibrotic targets. Neither p38 nor phosphorylated JNK demonstrated changes in expression upon addition of miR-18a mimic and phospho-p38 was not observed in the absence of TGF-β (Figure 3-9a). Surprisingly, ERK1/2 showed a robust ~ 50% decrease in its phosphorylation while the total ERK1/2 was the same in presence of miR-18a mimic (Figure 3-9b). The upstream kinase of ERK is MEK1/2 and MEK1/2 showed similar decrease in its phosphorylation (~ 30%) while total MEK1 was unchanged when miR-18a mimic was added (Figure 3-9b). This indicated that miR-18a is suppressing upstream signaling in the non-canonical pathway of TGF-β, affecting MEK1/2 phosphorylation which decreases ERK1/2 phosphorylation and not changing JNK and p38 levels or signaling.

Overexpressing miR-18a through addition of its mimic lowered smad2, CTGF expression and significantly decreased MEK1/2-ERK1/2 phosphorylation, effectively blocking transfer of TGF-β signaling through its downstream pathways. I then tested an in-vitro scratch assay to record migration of LX-2 cells after a scratch in a cell monolayer to mimic cell migration during wound healing in vivo (Liang, 2007), as a way to characterize the functional effect of the decreased TGF-β signaling. Migration after addition of negative control mimic was taken as baseline. 24 hours after scratch, miR-18a

mimic transfected cells had migrated significantly less (7%), compared to the control mimic (33%) transfected cells (Figure 3-10). Addition of miR-18a mimic did not affect collagen protein secretion or col1a1 transcript levels (Figure 3-14a, b), which correlates with the fact that no collagen genes are a direct target of miR-18a.

3.2.7 miR-18a down regulates canonical TGF-β signaling components even in presence of TGF-β

Overexpressing miR-18a had a profound effect on downstream TGF-β signaling and cell migration in-vitro. To accurately mimic in-vivo conditions, we overexpressed miR-18a mimic in the context of exogenous TGF-β addition in LX-2 cells. Since phosphorylation induced by TGF-β signaling is short-lived, protein was collected 2 hours and mRNA 24 hours after TGF-β addition. Mimic was added 24 hours prior to RNA/protein isolation for all further experiments.

When both miR-18a mimic and TGF-β were added together, miR-18a expression was increased (Figure 3-11a) and CTGF mRNA expression was still decreased by ~ 50% (Figure 3-11b), suggesting that miR-18a could destabilize CTGF mRNA transcript overruling transcriptional induction of CTGF mRNA by TGF-β (Chen, 2000). pSmad3 was expressed at baseline levels which were unchanged by addition of TGF-β, neither did smad4 (Figure 3-11c). Total Smad2 protein levels were also decreased by miR-18a even in the presence of active TGF-β signaling and this correlated with the decrease in phospho-Smad2 expression (Figure 3-11c) when TGF-β was added. pSmad2 (phospho-Smad2) cell specific expression was analyzed in whole liver tissue from alcoholic fibrotic rat livers including all the controls. Nuclear pSmad2 signal was observed in hepatocytes

in all groups including control diet fed animals, with nearly 100% of hepatocytes staining positive only in combination group (Figure 3-13a). Ethanol feeding has been documented to increase phosphorylation of Smad2 (Gerjevic, 2012). Interestingly, in the combination group with both ethanol and low dose CCl_4, negative staining for p-smad2 was only observed in spindle-shaped cells lining fibrotic septae (Figure 3-13b), the same location of miR-18a positive cells by in-situ (Figure 3-4b) and α-SMA positive cells in fibrotic livers (Figure 3-5b). This data suggests that miR-18a is down regulating its target Smad2 and pSmad2 both in-vitro in LX-2 cells and in-vivo in spindle-shaped stellate cells.

Non-canonical signaling proteins including p38 and total ERK1/2 were not changed and phospho-p38 was not expressed (Figure 3-12) when both miR-18a and TGF-β signaling were active. pERK1/2 down regulation as observed under miR-18a mimic vanished when TGF-β was added (Figure 3-12) while pMEK1/2 was still decreased even in presence of active TGF-β signaling. This is highly suggestive of alternate phosphorylation mechanisms of ERK1/2 under active TGF-β signaling.

3.2.8 miR-18a down regulates collagen even in the presence of active TGF-β signaling

Since Smad2 expression and activity and CTGF expression were down regulated by miR-18a even in presence of active TGF-β signaling, and miR-18a is expressed in stellate cells in fibrotic livers, we tested the effects of miR-18a, with or without TGF-β, on collagen production. Mimics and TGF-β were added to LX-2 cells and soluble collagen in supernatant was analyzed after 48 hours. Adding miR-18a mimic alone did not change collagen protein level but when TGF-β was added with negative control

mimic, collagen was increased by ~ 40%. Adding miR-18a mimic along with TGF-β decreased collagen levels back to levels of mimic without TGF-β (Figure 3.14a). Interestingly, Col1a1 gene expression showed no difference upon adding miR-18a in presence of active TGF-β signaling (Figure 3.14b), suggesting post-transcriptional mechanisms of miR-18a mediated down regulation of collagen.

3.3 Discussion

We developed an alcohol mediated fibrosis model in rats by introducing ethanol into their liquid diet and injecting them with intra-peritoneal CCl_4. This led to a profound fibrosis, steatosis, and necrosis in a short time period (6 weeks). We found that miR-18a, one of the miRNA in the miR 17-92 cluster is up regulated by 5-fold in the fibrotic animal livers only. This up regulation was partly transcriptional and partly post-transcriptional, although our evidence for the RNA processing chaperone, hnRNP A1, upregulating miR-18a in-vivo was inconclusive. In-situ for miR-18a revealed a localization specific to spindle shaped cells lining fibrotic septae in combination treated animals, α- SMA positive cells were also in the same location and spindle shaped as well, suggesting that the miR-18a positive cells are α–SMA^+ , although co-localization on the same slide is necessary to confirm this data. Non-parenchymal cells in the liver can be endothelial, Kuppfer, fibroblasts or stem cells. Since these cells are spindle shaped, they are definitely not of parenchymal origin, and since they are in the same location as α–SMA^+ cells, they are most likely fibroblasts.

To identify the functional role of up regulated miR-18a in fibroblast cells in the liver, we used LX-2 cells as surrogate primary stellate cells, in-vitro. TGF-β, one of the

major pro-fibrotic cytokines, which has been documented to be increased in most fibrosis, surprisingly down regulates miR-18a post-transcriptionally. This raises the question of how miR-18a is up regulated in fibrotic livers when it is down regulated by TGF-β in-vitro. Many possible explanations exist:

-other pathways/cytokines increase miR-18a (e.g. IL-6 up regulates miR 17-92; Brock, 2009)

-In-vitro results are not physiologically relevant.

- Downregulation of miR18a by TGF-β in individual cells is offset by the increase in number of cells which express miR-18a, reflecting an increase in whole tissue.

miR-18a mimic addition to LX-2 cells down-regulated its targets CTGF, Smad2 in the canonical pathway of TGF-β signaling. Additionally, miR-18a also decreased ERK1/2 phosphorylation, a novel result which has not been previously known. Correspondingly, upstream MEK1/2 phosphorylation was also decreased. Other components of non-canonical TGF-β signaling displayed no differences in protein expression upon adding miR-18a. Overall, overexpression of miR-18a led to a substantial reduction in TGF-β signaling, resulting in lowered migration of LX-2 cells in an in-vitro scratch assay. This functional phenotype is important since fibroblasts migrate to the site of injury and synthesize ECM proteins. miR-18a on its own did not affect collagen levels, indicating that active TGF-β signaling may be necessary to study some fibrotic effects.

In alcoholic fibrotic livers, TGF-β signaling is active and miR-18a is upregulated; we tried to replicate these two conditions in LX-2 cells. When both miR-18a mimic and TGF-β were added together, miR-18a was still able to down regulate its targets- CTGF and Smad2. pSmad2 was observed only when TGF-β was added; miR-18a decreased the

amount of pSmad2, suggesting that canonical TGF-β signaling was still decreased in presence of exogenous TGF-β. In contrast, the downregulation of pERK observed when miR-18a was added, was abolished by the addition of TGF-β. More importantly, the other non-canonical component, p38 showed no increase in expression, to compensate for the decrease in canonical TGF-β signaling. This is highly suggestive of overall downregulation of TGF-β signaling when miR-18a and TGF-β is added together. Consistent with this reasoning, post-transcriptional collagen production was decreased in LX-2 cells, with the co-addition of TGF-β and miR-18a. miR-18a by itself could not decrease collagen mRNA or protein, but since it downregulates upstream TGF-β signaling, it leads to reduced collagen in presence of exogenous TGF-β . Similarly, pSmad2 IHC revealed that some of the same spindle shaped cells (fibroblasts) are signal negative, indicating that the decrease in pSmad2 we saw in LX-2 cells is also recapitulated in-vivo.

miR-18a directly targets many genes in TGF-β signaling pathway, including Smad2, 3, 4 and CTGF. This makes it a powerful anti-fibrotic molecule, since downregulation of each of these targets is anti-fibrotic. For instance, inhibition of CTGF prevented liver fibrosis in CCl_4 (Li, 2006), and in *N*-nitrosodimethylamine-induced (George and Tsutsumi, 2007) injury. CTGF is also in the pipeline for drug development, for use in treating kidney, lung and liver fibrosis by FibroGen, Inc. with phase 1 clinical trials already completed. Disruption of Smad4 inhibited renal fibrosis in-vivo by blocking TGF-β signaling and inflammation (Meng, 2012).

The conclusion from our data is summarized in Figure 3.15. During injury to the liver from alcohol and CCl_4, both miR-18a and TGF-β are induced, acting on the same

cell type known to produce ECM proteins, fibroblasts or stellate cells in the liver. TGF-β downregulates miR-18a and miR-18a downregulates canonical and non-canonical TGF-β signaling and this mutual inhibition results in a lessening of the fibrotic phenotype i.e. collagen production and stellate cell migration is reduced. miR-18a plays a powerful protective role in alcohol mediated fibrosis, down regulating TGF-β signaling and downstream fibroblast activity.

From our results, we could hypothesize that miR-18a could be a prognostic indicator of the severity of fibrosis. In early stages of fibrosis, upregulated miR-18a is protective. As fibrosis proceeds and beyond a certain point, miR-18a should be downregulated in accordance with increased TGF-β activity. Consistent with this theory, miR-18a has been shown to be increased at day 2 after acute myocardial infarction in the heart but was back at normal levels by day 7 (Shi, 2010). miR-18a has also been documented to be decreased in severe fibrosis, specifically in myocytes in aging heart (van Almen, 2011), and the miR 17-92 cluster is decreased in the lung of IPF patients (Dakhlallah, 2013).

3.4 Conclusions

In this chapter, I enumerate the protective roles of miR-18a in an alcohol and CCl_4 mediated fibrotic model. miR-18a is increased in fibrotic animal liver and in-situ showed the differential expression in fibrotic animals is limited to spindle shaped cells lining fibrotic septae, which are the same location as α-SMA positive cells. miR-18a directly targets CTGF and Smad2, and indirectly down regulates non-canonical TGF-β signaling. pSmad2 signal was reduced in the same spindle shaped cells lending further proof to miR-

18a mediated down regulation of TGF-β signaling in fibroblasts. TGF-β, in turn post-transcriptionally decreases miR-18a. The mutual inhibition of miR-18a and TGF-β signaling could reflect a constant balance of activation and inhibition of pro-fibrotic signaling, resulting in alteration of key fibrotic phenotypes such as fibroblast migration and collagen synthesis. miR-18a counteracts TGF-β induced pro-fibrotic signaling and is protective in alcohol mediated liver fibrosis.

3.5 Materials and methods

3.5.1 Fibrosis model in rat: alcohol and CCl_4

Lewis rats were divided into four groups and all diets were followed for 6 weeks: 1) Isocaloric control and 2) 7% ethanol liquid diets following the Lieber-DeCarli regimen (BioServ) 3) control plus 0.1ml/kg body weight carbon tetrachloride (CCl_4, Sigma-Aldrich, Inc.) intraperitoneal injections twice a week, and 4) a combination group of ethanol plus low dose carbon tetrachloride. The Johns Hopkins School of Medicine Animal Care and Use Committee approved all housing and procedures. 5 μM sections of formalin fixed, paraffin embedded liver samples were cut and stained with hematoxylin and eosin as well as Masson's trichrome by the Johns Hopkins Reference Histology Lab (Baltimore, MD).

Total liver RNA was prepared using a mirVana miRNA Isolation Kit (Ambion, Inc, Austin, TX) according to the manufacturer's protocol. 500ng RNA was reverse transcribed without pre-amplification using Megaplex RT Primers (Rodent Pool A v2.0; Applied Biosystems) and a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's protocol. The microarray was run on the

7900HT Fast Real-Time PCR System with TaqMan Array Rodent MicroRNA A Cards v2.0 (Applied Biosystems), according to the manufacturer's protocol. The geometric mean of each plate was used for normalization and the top 50 miRNAs with the greatest average fold change over the control diet, sorted by the combination group (both alcohol and low-dose CCl_4) were drawn as a heat map using R, a statistical programming language.

Total RNA was isolated from cultured cells or whole liver tissue using Trizol (Invitrogen) according to the manufacturers' protocols. Expression of 18s rRNA, mature miR-18a, miR-106b and mature miR-17 was assessed using individual Taqman assays, with 18s used as normalizing control (Applied Biosystems). Quantitative PCR using sybr green (Life technologies) was done using manufacturer's instructions with primers and β–actin was used as normalizing internal control.

3.5.2 Histological staining

Immunofluorescence. Liver tissue samples were fixed in fixed in 10% formalin overnight and embedded in paraffin. 5 μm sections were transferred to Superfrost/Plus Microscope Slides (Fisher Scientific) and allowed to dry at 60 °C for 18 minutes. Slides were deparaffinized using standard techniques and blocked with PBS + 5% fetal bovine serum (Sigma-Aldrich, Inc.) Slides were then incubated in the dark for 1.5 hours at room temperature with a conjugated mouse α-SMA-cy3 antibody (Sigma-Aldrich, Inc.) or rabbit hnRNP A1 antibody (Novus Biologicals) and washed three times in PBS. Secondary antibody anti-rabbit IgG Alexa Fluor 488 conjugate (Cell Signaling Technology) was added for hnRNP A1 only and incubated for an hour in the dark. Then,

the slides were counterstained with DAPI (Life Technologies) and mounted using a fluorescence mounting medium (DAKO).

In-Situ Hybridization (ISH) ISH was performed on 5 μm RNAse-free slides from the rat fibrotic model four groups using Exiqon's FFPE (Formalin fixed paraffin embedded) kit. Locked nucleic acid (LNA) detection probes for has-miR-18a and a scrambled negative control were from Exiqon. The assay was performed according to the manufacturer's instructions except for the following change- proteinase k was used at a final concentration of 13.8 μg/ml.

Immunohistochemistry Slides (6 μm) were deparaffinized and antigen retrieval was completed using a steamer. IHC for pSMAD2 (Millipore) was performed according to the manufacturer's instructions and developed colorimetrically using DAB peroxidase substrate kit (Vector Labs), and counterstained with hematoxylin.

3.5.3 In-vitro experimental setup.

Addition of cytokines to cells/Transfection Cells were plated with normal media (DMEM (Life Technologies) with 10% Fetal bovine serum), switched to 5% serum the next day and 10 ng/ml TGF-B (Roche) was added 24 hours later. RNA was collected after an additional 24 hours. LX-2 cells were plated in a 6 well plate and 24 hours later, 10 μm hsa-miR-18a-5p mimic and mirVana miRNA mimic negative control #1 mimic (Life Technologies) was added using Lipofectamine RNAiMAX reagent in Opti MEM media (Life Technologies). RNA was isolated using Trizol (Life Technologies) 24 hours later. ScAAV8.miR-18a.eGFP or control plasmid were transfected into LX-2 cells and analysed for GFP expression 24 hours later.

Western blot LX-2 cell lysates were run on a gel, and probed with antibodies for Smad2, pSmad2, ERK1/2, pERK1/2, pSmad3, Smad4, p38, MEK1/2, MEK1/2 (Cell Signaling Technologies), pJNK1/2 (Santa Cruz Biotechnology Inc.). Blots were stripped (Newblot PVDF stripping buffer, Licor) and reprobed for b-Actin (Sigma-Aldrich Inc.)

Scratch assay Scratch assay was performed using the protocol as described in Liang et al, 2007. Briefly, LX2 cells were plated onto poly-L-lysine coated plates. The plates were replaced with 5% FBS +DMEM media and 24 hours later, transfected with 10 nM miR-18a or negative control mimic. The next day, the plate was scratched with a p200 tip and washed. Brightfield images were taken at this time point t=0; 24 hours later, the plate was imaged again to detect migration of cells. Atleast 24 images were taken per condition at every timepoint and area under the curve (between the two ends of the scratch) was measured using ImageJ (NIH).

Collagen Assay LX2 cells were plated on normal media and switched to 5% FBS the next day. 24 hours later, mimic transfections and/or TGF-β addition was performed. 48 hours later, the cell supernatant was collected and concentrated. Cell free media with the same serum concentration was also collected to function as baseline controls. Collagen levels were determined by Sircol Collagen Assay (Biocolor), which was performed according to the manufacturer's protocol.

scAAV8.miR-18a.eGFP construction hsa-miR-18a was cloned into the locus of miR-26a in scAAV.miR-26a.eGFP (Kota, 2009). Three constructs were made of successively longer lengths outside of the miR-18a locus, encompassing parts of miR-17 on the 5' end and miR-19 on the 3' end. All plasmids were sequenced to verify the locus of insertion and lack of mutations.

3.6 Tables: Chapter 3

Table 3-1. List of targets of miR-17-92 cluster in TGF-β pathway

Target	Targeted by	Conserved	Process	PMID
TGFBR2	mir-17, 20a, 19a	yes	TGF-β signaling	22095742, 22278637
Smad2	mir-18a	yes	TGF-β signaling	22095742
Smad4	mir-18a	no	TGF-β signaling	22095742
CTGF	mir-18a, 19a	yes	TGF-β signaling, angiogenesis	23249750
Smad3	mir-18a	No	TGF-β signaling	23249750
Tsp1	mir-18a	No	TGF-β processing	21402698

Target genes, and their lack of conservation between rodents and humans is indicated in column 1 and 4. PMID denotes the Pubmed reference ID for publications with luciferase assays proving TGF-β pathway genes are targeted by miR 17-92 cluster miRNAs.

Table 3-2. Fold change of miR17-92 and family members in alcohol mediated fibrosis.

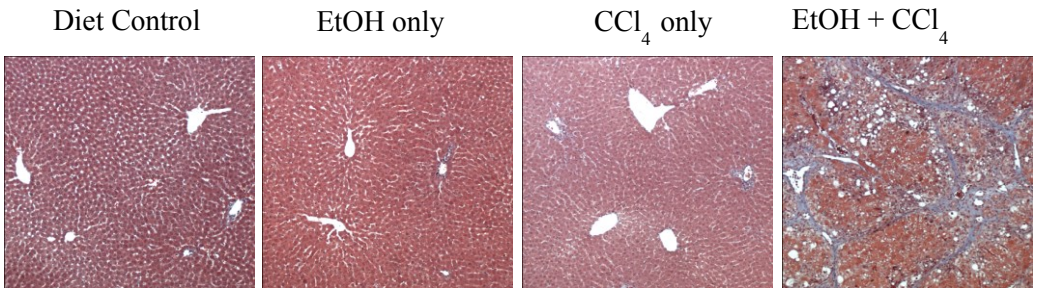
mirna	fold change (combo/control diet)
mmu-miR-17	3.4002
rno-miR-17-3p	8.7845
mmu-miR-18a	30.5127
mmu-miR-19a	consistent expression across groups
mmu-miR-20a	2.2374
mmu-miR-19b	consistent expression across groups
mmu-miR-92a	consistent expression across groups
mmu-miR-106a	3.6256
mmu-miR-18b	not expressed
mmu-miR-20b	4.3875
mmu-miR-363	Not expressed
mmu-miR-106b	3.2632
mmu-miR-93	4.1963
mmu-miR-25	2.5022

3 out of the 6 members of miR17-92 family showed up regulation in the combination treated animals as compared to control diet treated animals. miR106b-25 cluster miRNAs (106b, 93 and 25) also showed consistent up regulation.

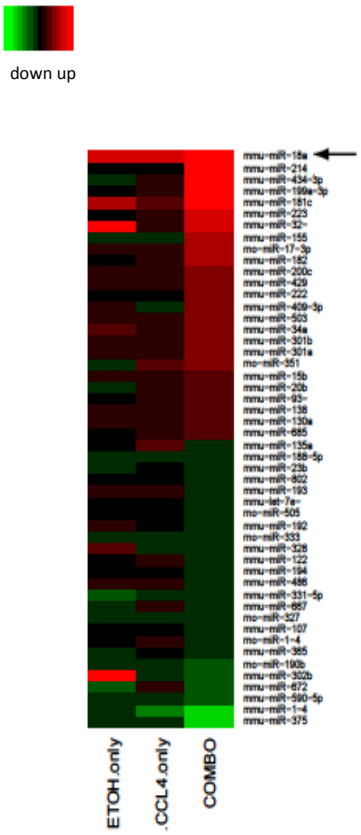
3.7 Figures: Chapter 3

Figure 3-1. miR-18a is upregulated in alcohol-mediated fibrosis.

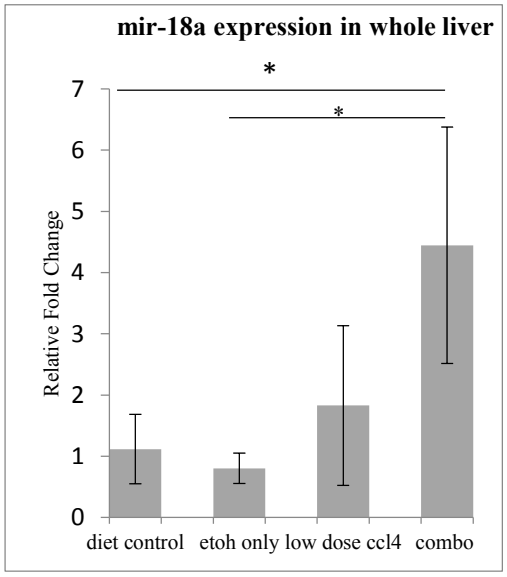
a



b

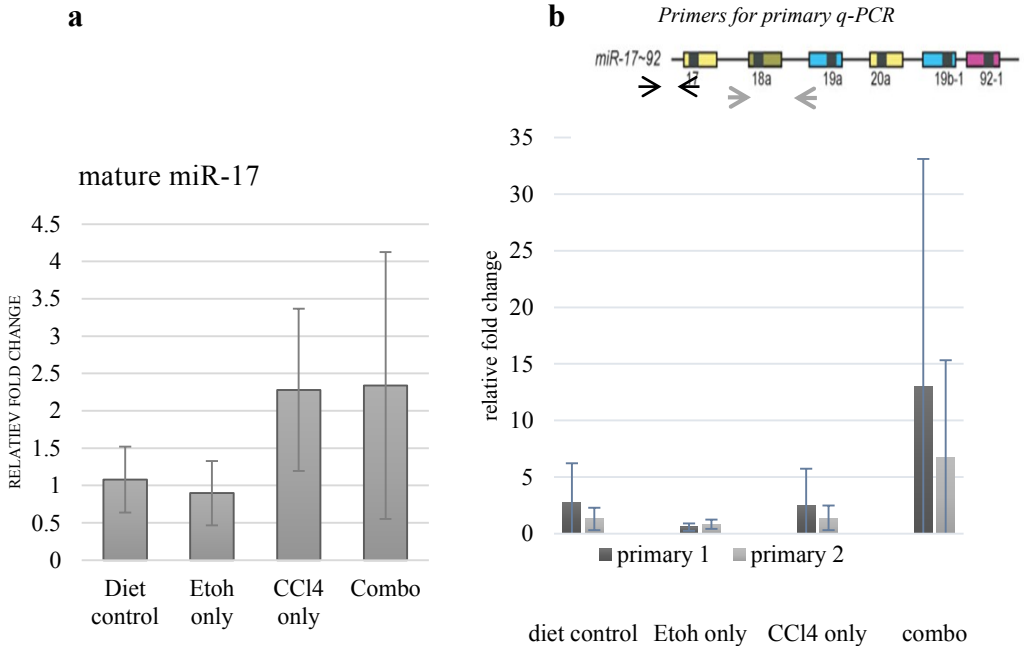


c



(a) Representative trichrome (collagen stains blue) images of liver from animals in the four groups of alcohol mediated fibrosis (b) Heatmap of top 50 most changed miRNAs, normalized to the control diet group and sorted by the combination group with arrow indicating top most changed, miR-18a (c) Verification of array data by individual taqman of miR-18a in the four groups, normalized to control diet group animals; n=5 in all groups except combo, n=4.

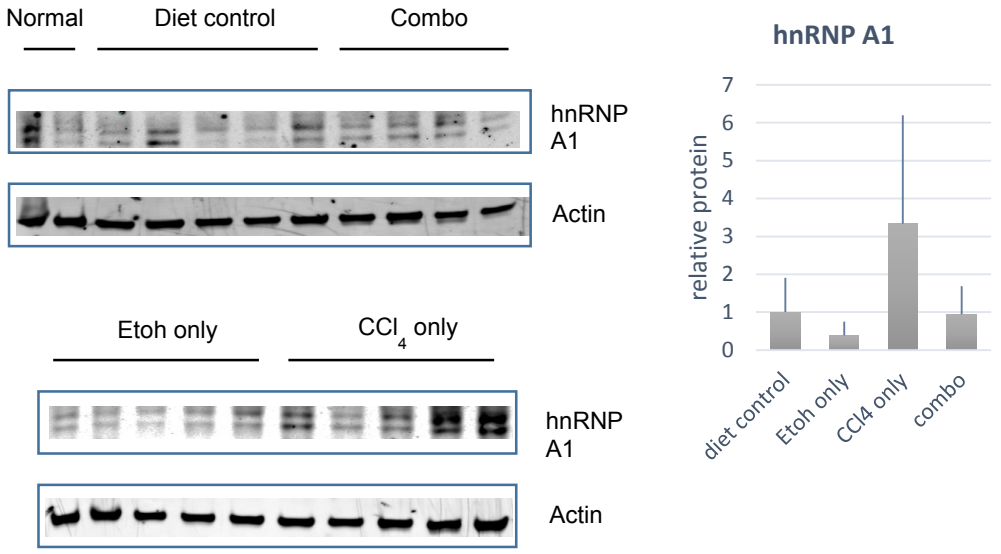
Figure 3-2. Transcriptional up regulation of miR-18a in alcohol mediated fibrosis



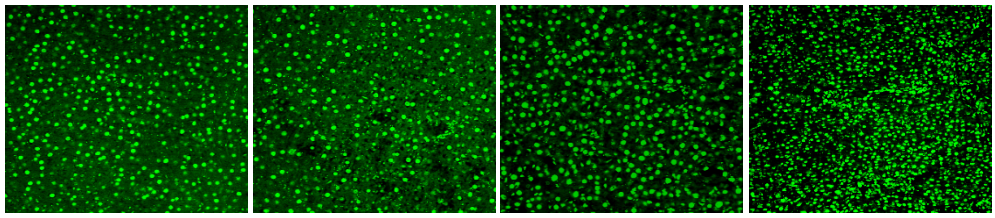
(a) miR-17 expression by taqman in the four groups of alcohol mediated fibrosis. (b) Primary transcript expression of miR-17-92 was analyzed by Q-PCR using two primer sets (indicated as black (1) and gray (2) in miR17-92 map).

Figure 3-3. hnRNP A1 expression and localization is unchanged during fibrosis.

a

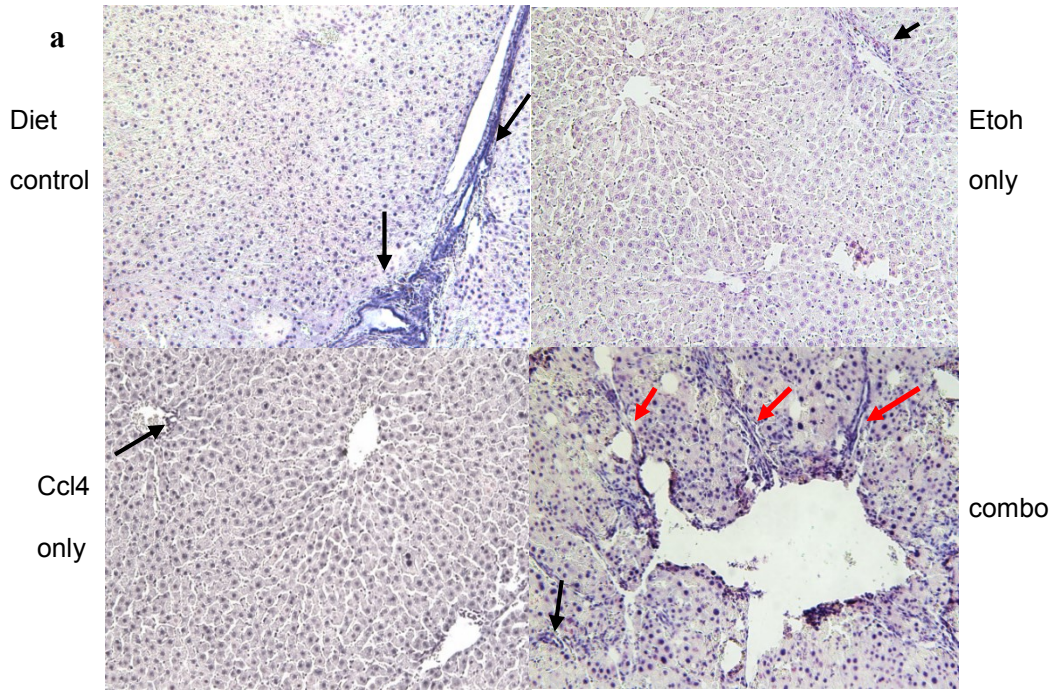


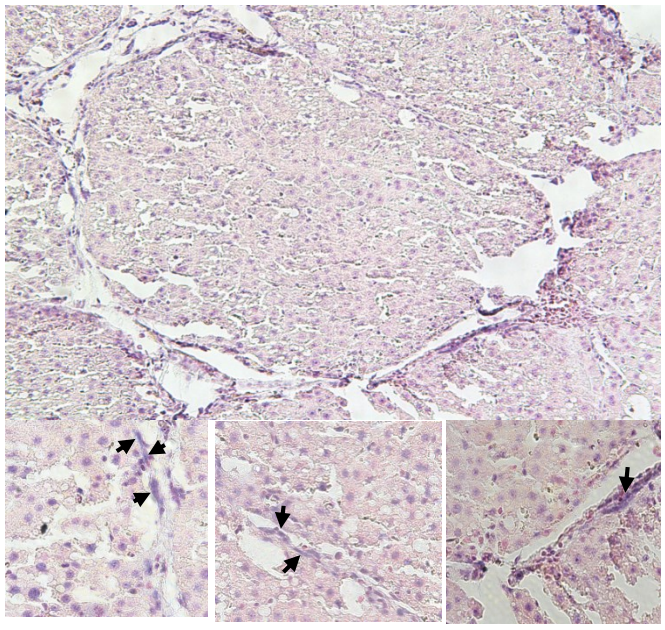
b



(a) Western blot for hnRNP A1 with actin as loading control; quantification of band intensity, normalized to actin probed in the same blot. (b) Representative immunofluorescence images for hnRNP A1 (green) in livers from four groups of alcohol mediated fibrosis. hnRNP A1 is nuclear in all animals. LD= low dose.

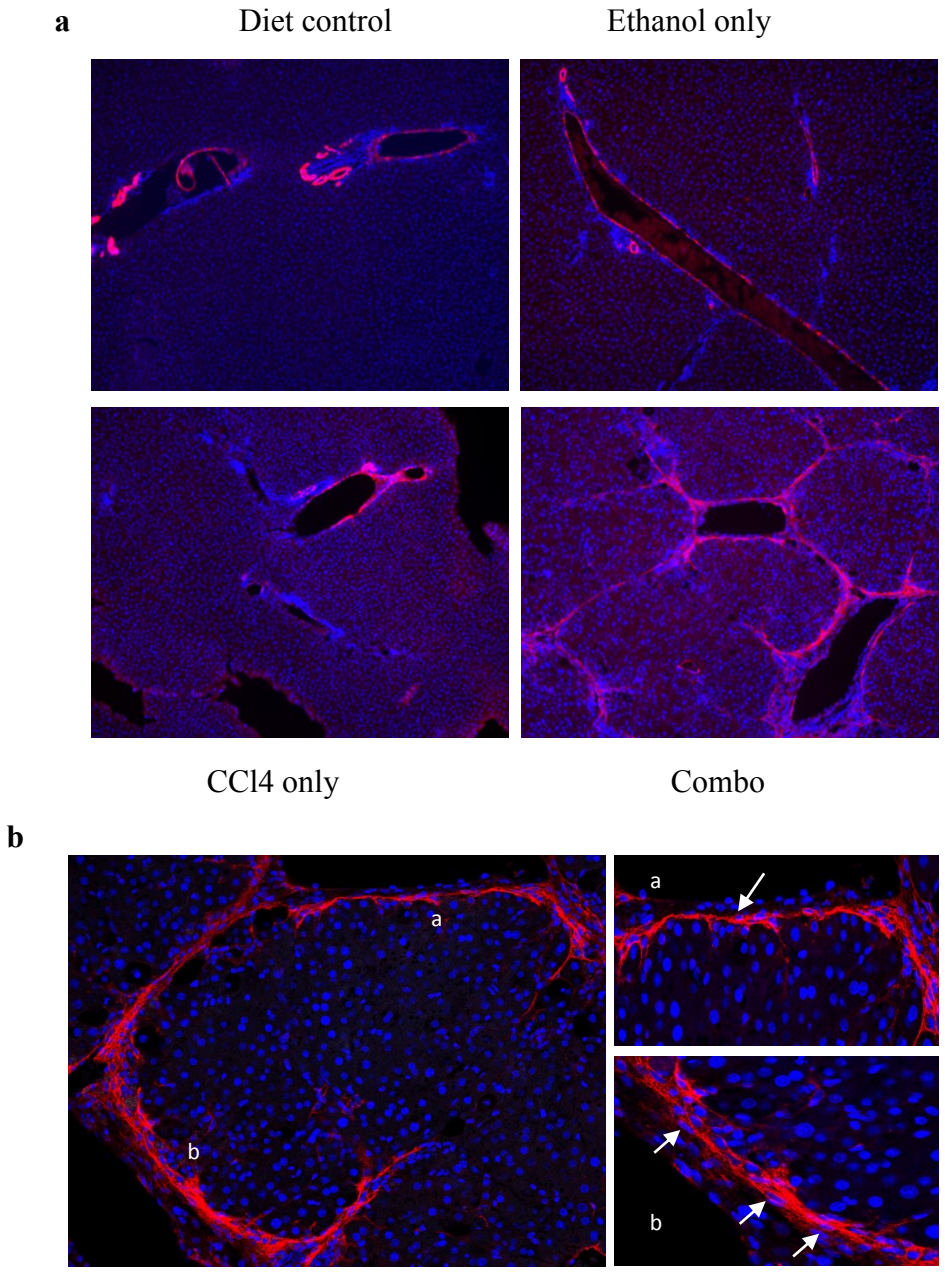
Figure 3-4. In-situ for miR-18a reveals positive spindle shaped cells along fibrotic septae, specific to fibrotic livers





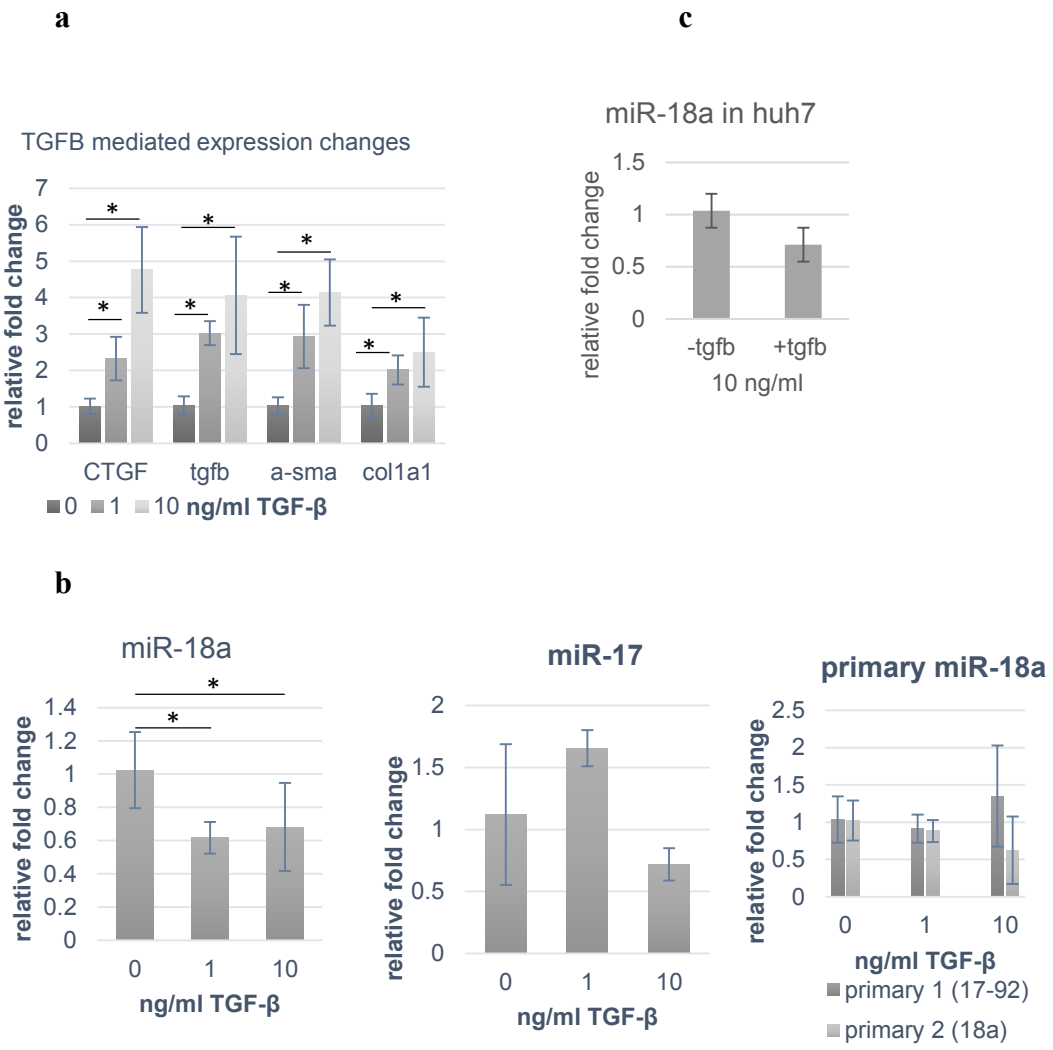
(a) Representative low magnification images from In-situ hybridization of miR-18a in the four groups- black arrows point to bile duct cells and red arrows point to positive (blue) signal in fibrotic septae in combo treated animals. (b) Top panel is low magnification image of a pseudo-lobule with areas of higher magnification (bottom panel of images) indicated. Black arrows point to spindle shaped cells

Figure 3-5. α-SMA positive cells are spindle shaped along fibrotic septae



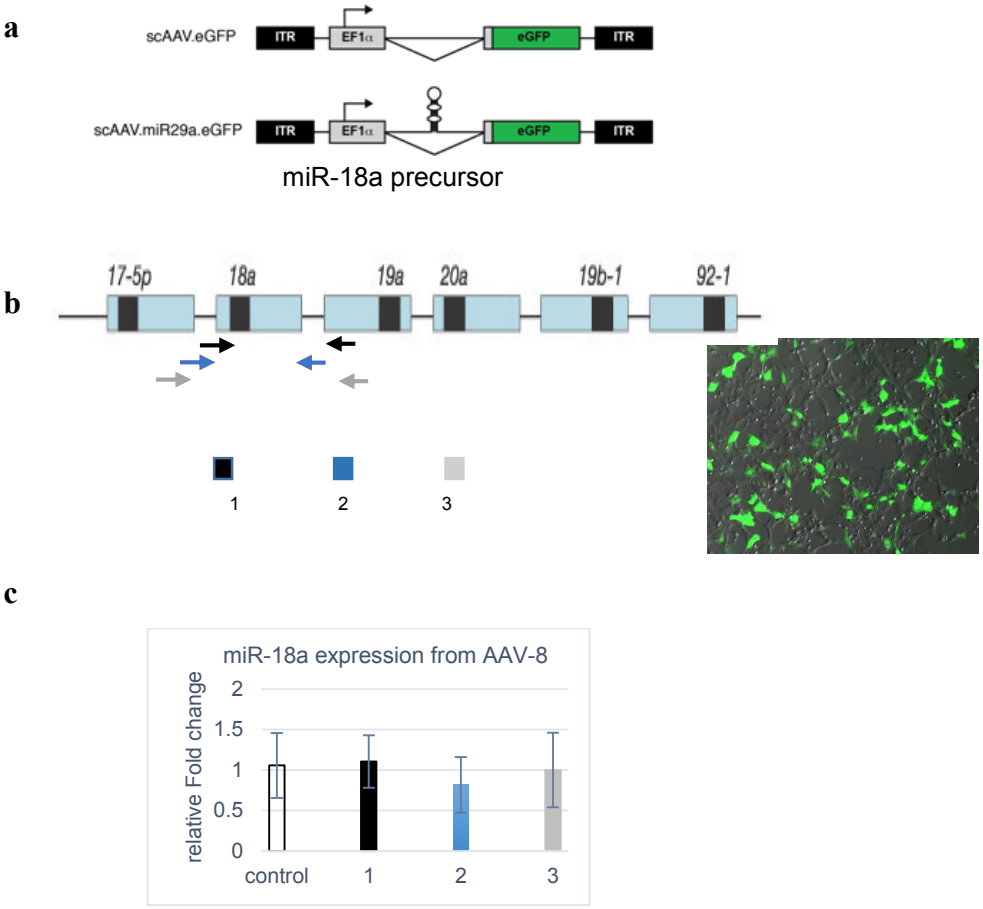
(a) Representative low magnification images from immunoflourescence of –SMA (red) in the four groups, counter-stained with DAPI. (b) Left panel is low magnification image of a pseudo-lobule with areas of higher magnification (right panel of images) indicated. White arrows point to spindle shaped cells

Figure 3-6. TGF-β downregulates miR-18a expression in-vitro



(a) Pro-fibrotic gene expression changes mediated by 1 or 10 ng/ml TGF-β addition to LX-2 cells, by Q-PCR. (b) miR-18a, miR-17 expression by taqman and primary miR-17-92 by Q-PCR with 2 different primer sets, assayed after addition of 1 or 10 ng/ml TGF-β

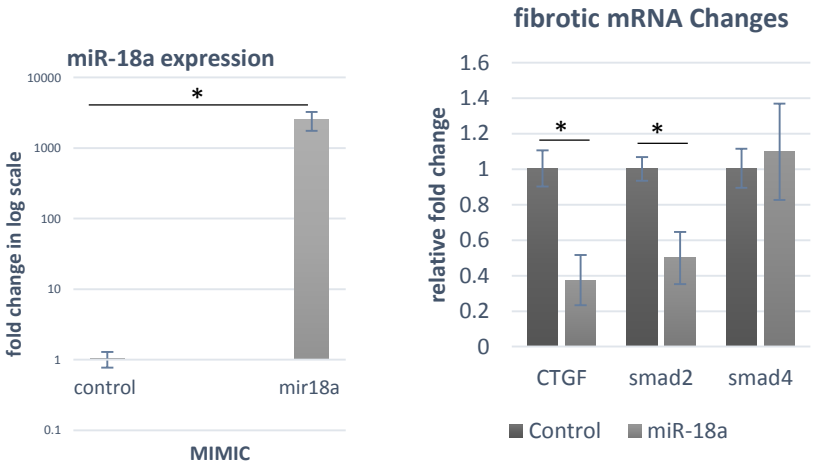
Figure 3-7. AAV8-miR-18a doesnot overexpress miR-18a in-vitro



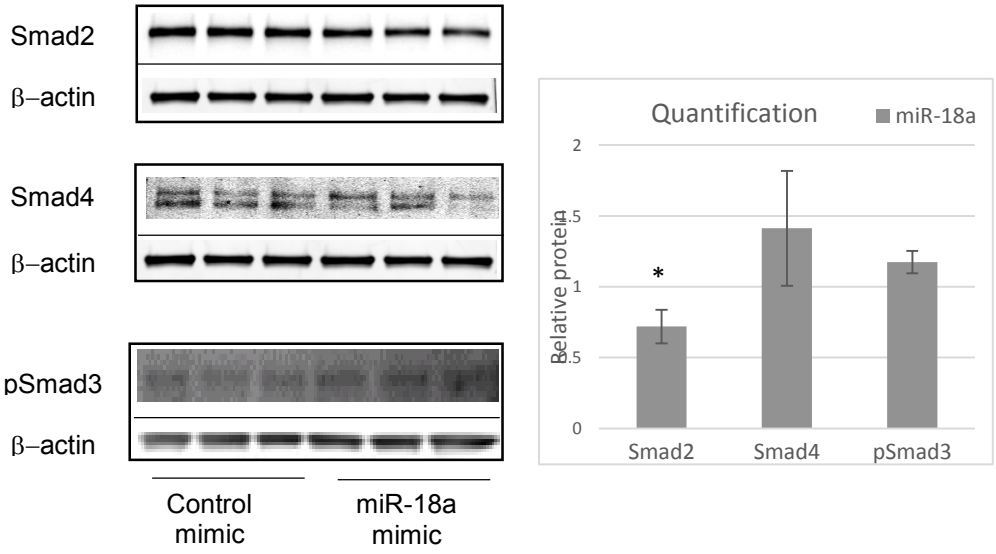
(a) Schematic representation of scAAV vectors depicting locations of inverted terminal repeats (ITRs), elongation factor 1-alpha promoter (EF1α), miRNA (shown in hairpin form), and enhanced green fluorescent protein (eGFP) open reading frame. (b) Diagrammatic map of primer sets (1, 2, and 3 in different colors) used for cloning increasing lengths of miR-18a into AAV-8, black shading indicates mature miRNA sequence in blue precursor box. Representative image of plasmids AAV8-miR-18a transfected into LX-2 cells showed virus expression by GFP (green) (c) Taqman quantification of miR-18a from the three different color-coded plasmids

Figure 3-8. miR-18a downregulates CTGF and Smad2 in LX-2 cells

a

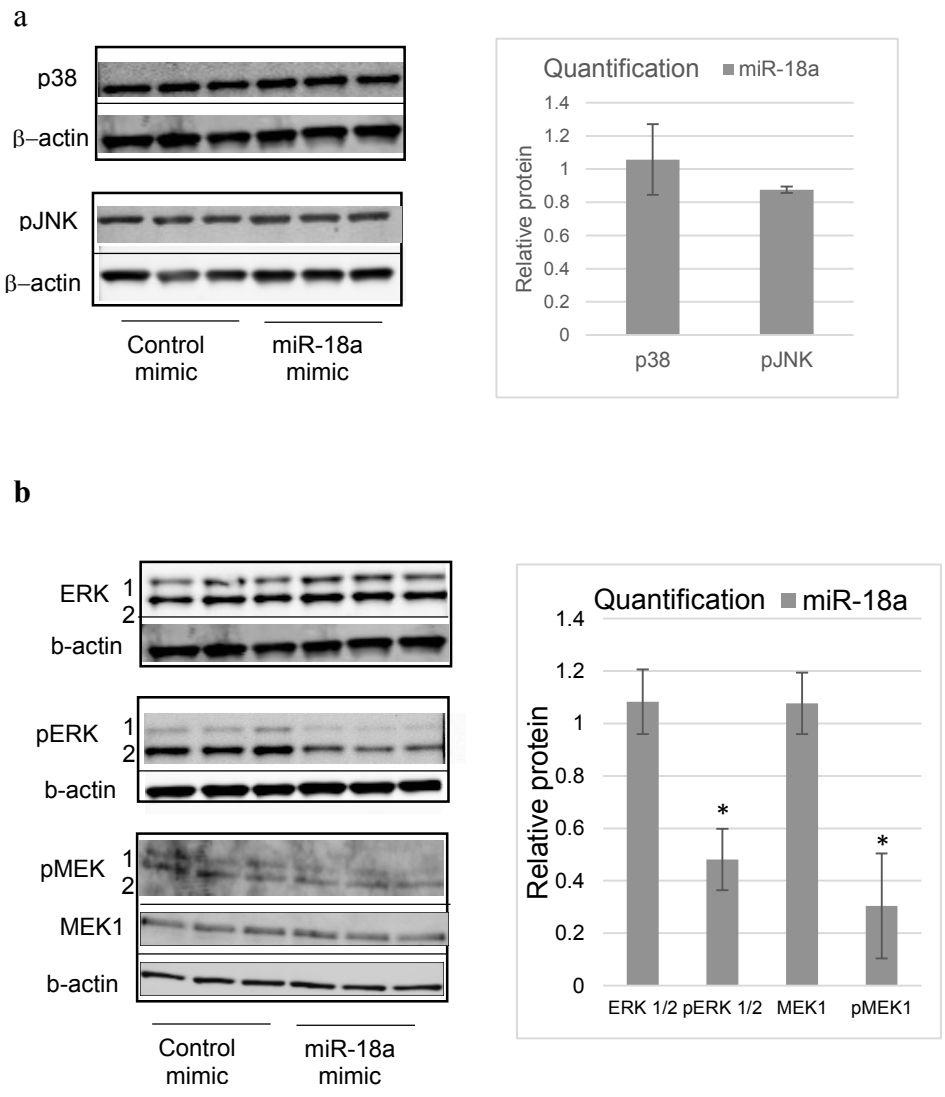


b



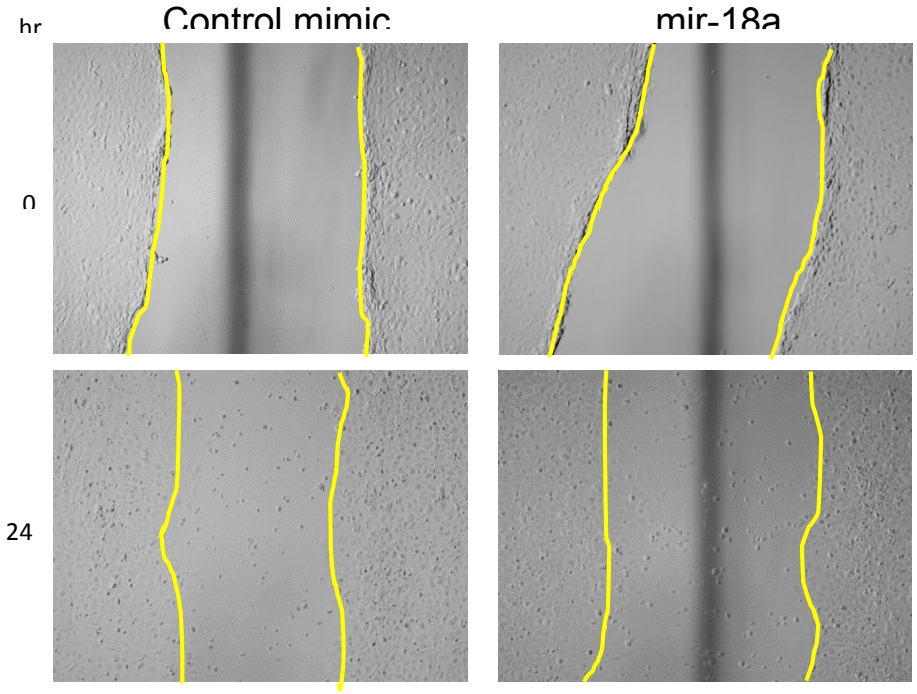
(a) miR-18a expression quantified after addition of 10 μM negative control or miR-18a mimic; note the log scale in Y axis. Quantification of fibrotic gene expression changes under the same conditions (b) Western blot of LX-2 cells transfected with 10 μM control or miR-18a mimic, probed and quantified for Smad2, Smad4 and phospho-Smad3 (pSmad3). Actin was the loading control, probed on the same blot. Graphs show only miR-18a mimic transfected group with control cells assumed to be 1.

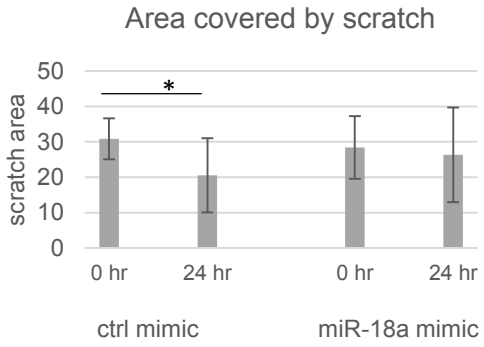
Figure 3-9. miR-18a downregulates components of non-canonical TGF- β signaling



Western blot of control and miR-18a transfected LX-2 cells, probed and quantified for (a) p38, phospho-JNK (pJNK), (b) ERK1/2, phospho-ERK1/2 (pERK), MEK1/2, and phospho-MEK1/2. Actin was used as loading control and in quantification of band intensity. Graphs show only miR-18a mimic transfected group with control cells assumed to be 1.

Figure 3-10. miR-18a delayed migration of LX-2 cells in a scratch assay

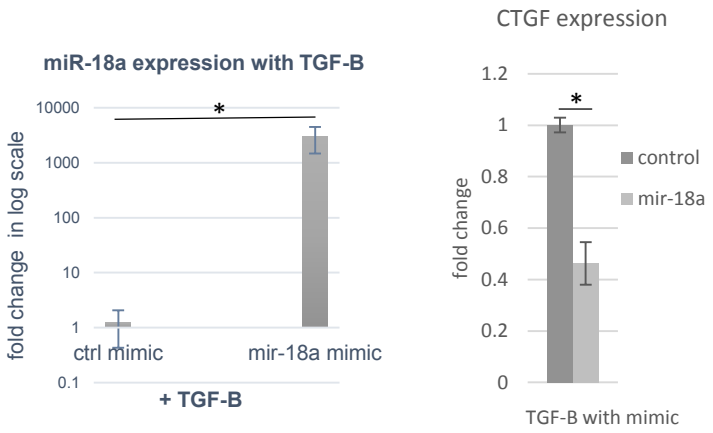


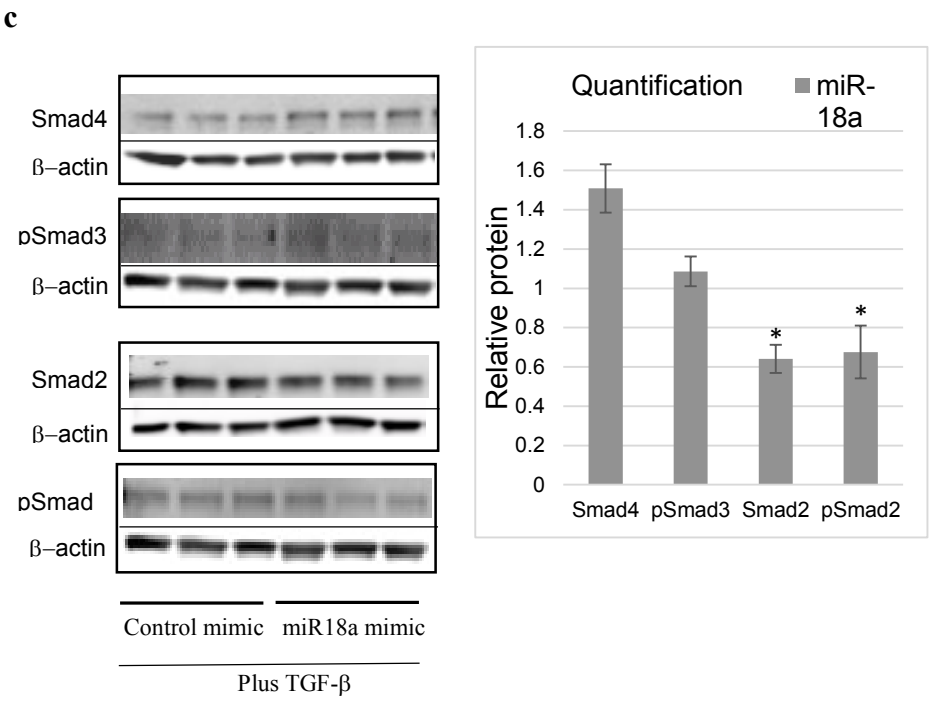


Representative images at 0 and 24 hours after a scratch on LX-2 cell monolayer, transfected with control or miR-18a mimics. Yellow lines denote boundary of scratch, and is the area counted in graph.

Figure 3-11. miR-18a downregulates canonical TGF-β signaling components even in the presence of exogenous TGF-β

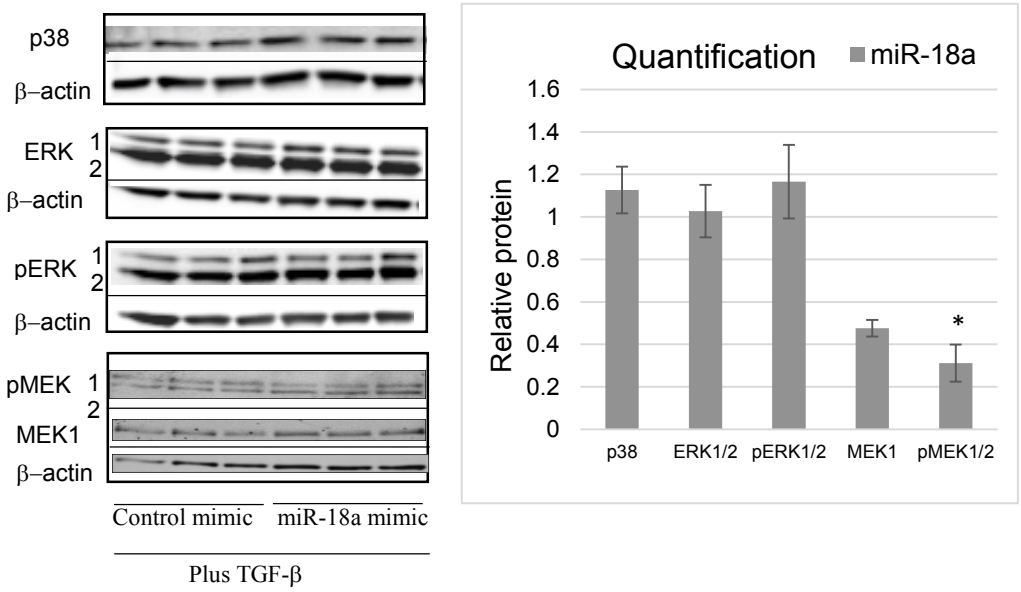
a **b**





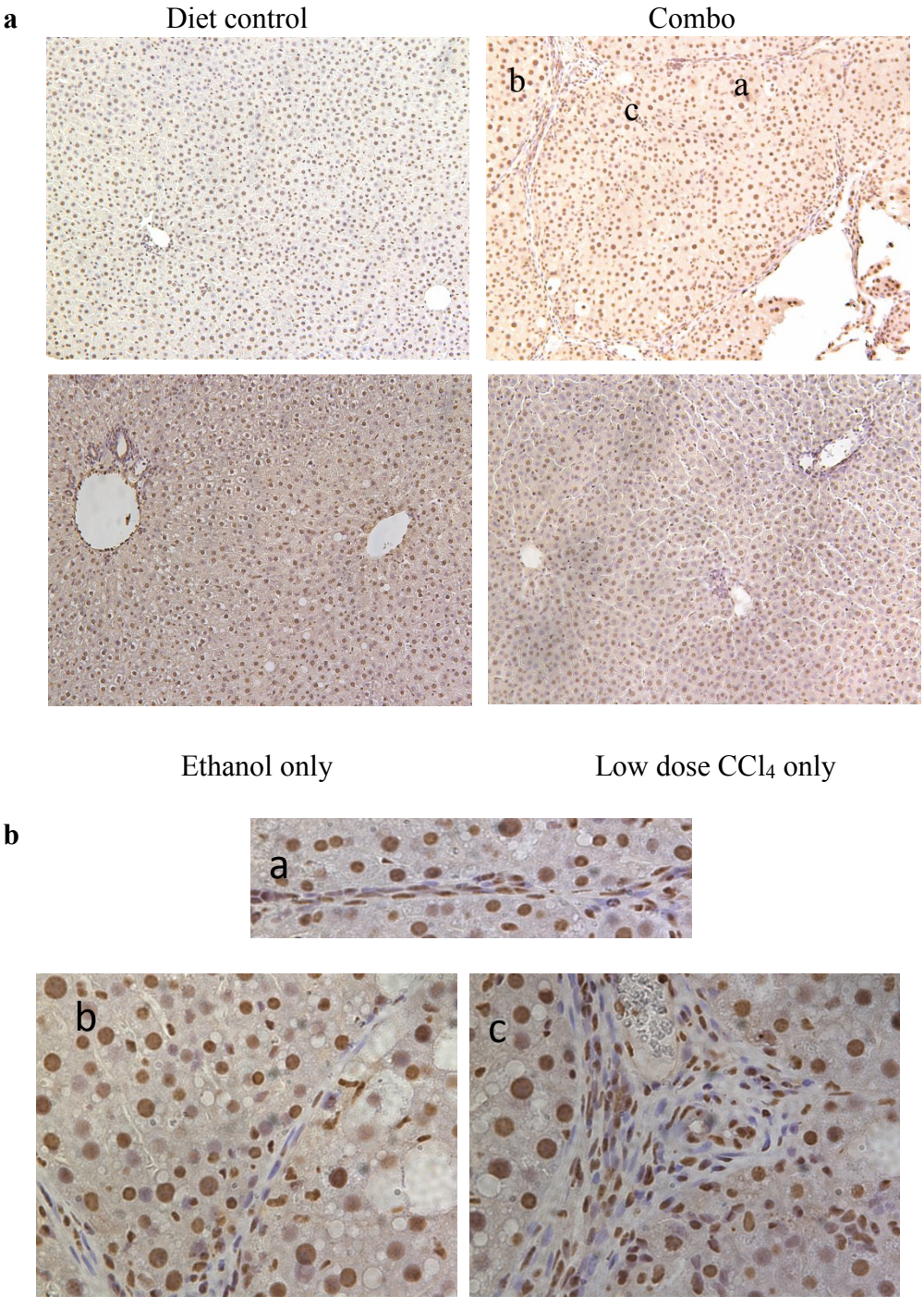
(a) Quantification of miR-18a expression by taqman (b) CTGF mRNA expression change quantified by Q-PCR, western blot probed for (c)Smad4, Smad2, pSmad2, and pSmad3 and quantified using the loading control, actin - after co-transfection with TGF-β and control or miR-18a mimics in LX-2 cells. Graphs show only miR-18a mimic transfected group with control cells assumed to be 1.

Figure 3-12. miR-18a regulation of non-canonical TGF-β signaling is abolished in presence of exogenous TGF-β



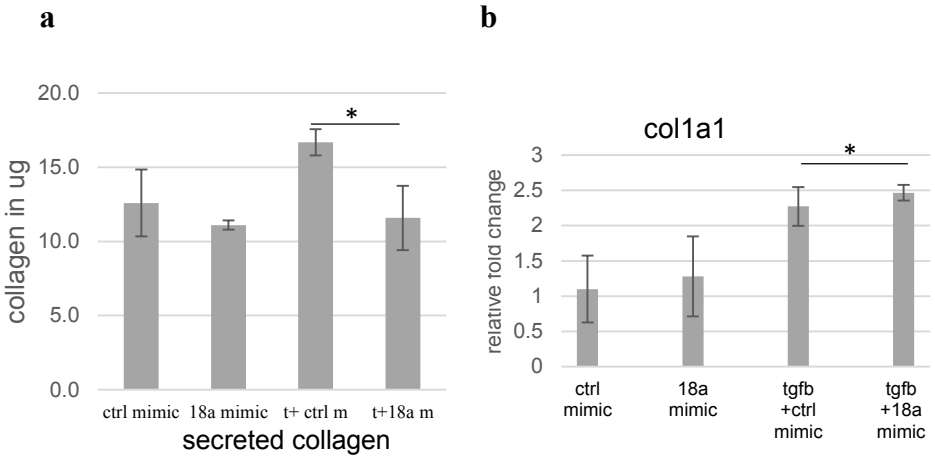
Western blot of LX-2 cells co-transfected with TGF-β and control or miR-18a mimic. Blots were probed for ERK1/2, pERK1/2, MEK1/2, pMEK1/2, and p38 with actin as loading control. Quantification of band intensity was normalized with actin on the same blot. Graphs show only miR-18a mimic transfected group with expression in control cells assumed to be 1.

Figure 3-13. pSmad2 signal is down regulated only in spindle-shaped cells lining fibrotic septae in alcohol mediated fibrosis



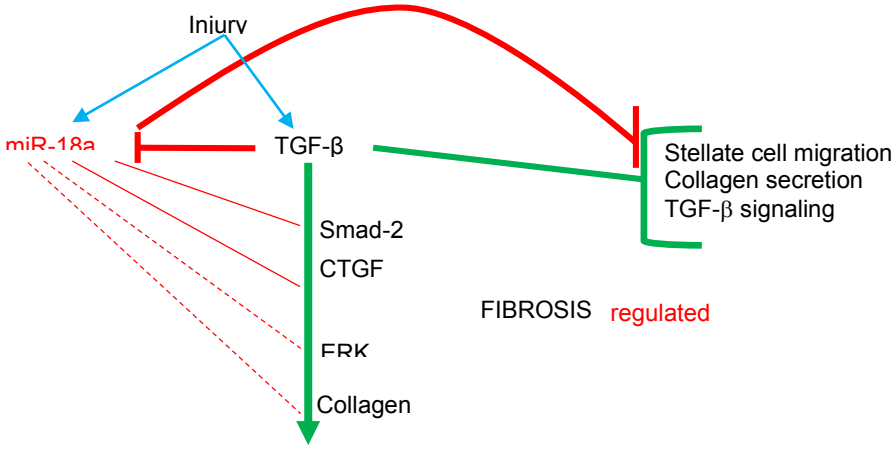
(a) Representative low magnification images of immunohistochemistry (IHC) for pSmad2 on whole liver from four groups of alcohol mediated fibrotic animals (refer figure 3.1a) with areas of higher magnification (b) indicated in fibrotic, combo treated animals. Brown is positive signal, counterstained with blue hematoxylin.

Figure 3-14. miR-18a downregulates collagen in presence of exogenous TGF-β



(a) Quantification of secreted collagen (in supernatant) from LX-2 cells transfected with control or miR-18a mimics and with and without co-addition of TGF-β. (b) Quantification of col1a1 gene expression by Q-PCR under the same conditions.

Figure 3-15. model for the regulatory role of miR-18a in liver fibrosis



In response to liver injury, both TGF-β signaling and miR-18a are up regulated. TGF-β, in turn activates its downstream signaling molecules (CTGF, collagen and Smad2, ERK phosphorylation) and also down regulates miR-18a. miR-18a counteracts TGF-β signaling by decreasing predicted target proteins (red unbroken line) and non-targets (ERK, collagen in broken red lines) and reduces the fibrotic phenotype of stellate cell migration, collagen production induced by TGF-β . Thus, miR-18a neutralizes the pro-fibrotic effects of TGF-β.

4. CHAPTER 4. CONCLUDING REMARKS

Liver fibrosis is a major clinical problem with no existing therapy to treat the underlying fibrotic mechanisms. We set out to identify and characterize the role of two miRNAs in fibrosis of the liver. miR-29a plays a powerful role in regulating fibrosis by downregulating the last step of ECM (extracellular matrix) production. We developed a therapeutic delivery of miR-29 method which successfully reversed established fibrotic phenotype. This illustrated the major role miR-29 plays in regulating fibrosis and delineates the power of using miRNAs as therapeutics. Another miRNA, miR-18a, in contrast, targets upstream pro-fibrotic TGF-β signaling pathway components. miR-18a is upregulated in alcohol mediated liver fibrosis and localizes specifically to cells in the same region as fibroblasts. Our data is highly suggestive that miR-18a plays a protective role during fibrosis by downregulating TGF-β signaling components and affecting fibrotic processes such as fibroblast migration and collagen secretion. This research highlighting the promising protective roles of both miR-29 and miR-18a supports and encourages the clinical development of diagnostic markers or therapeutic drugs in the near future.

4.1 Therapeutic delivery of miR-29a prevents and reverses liver fibrosis

Chapter two recounts the discovery of downregulation of miR-29 in a liver toxin induced fibrosis. miR-29a targets many proteins that are part of the extra-cellular matrix (ECM) and has long been known to be decreased in multiple fibrotic organs. We adapted a previously developed model for adding back miR-29 to fibrotic mice, using AAV (adeno-associated virus) vector. Surprisingly, in contrast to other miRNAs, miR-29 did

not show increased expression with the vector, suggesting a tightly controlled regulation of expression under physiological conditions. Additionally, AAV transduced hepatocytes predominantly and not the ECM producing stellate cells, the fibroblasts of the liver. Still, the virus was successful in preventing fibrosis in mice, both histologically and in preventing collagen production. The viral delivery system also showed strong reversal of ongoing fibrosis, suggesting that miR-29 can play a curative role in the clinic, treating patients with established fibrosis.

4.2 miR-18a is increased in alcoholic liver fibrosis and regulates TGF-β signaling

In chapter three I describe the upregulation of miR-18a in a model of alcohol and hepatotoxin induced fibrosis. Upregulated miR-18a is localized specifically to spindle-shaped cells in the same region as α-SMA positive stellate cells. In-vitro, miR-18a regulates its sequence based targets such as Smad2 and CTGF, components of the canonical arm of TGF-β signaling pathway and also downregulates ERK phosphorylation, a key non-canonical signaling component of TGF-β. miR-18a slowed migration of stellate cells in-vitro and also post-transcriptionally downregulated downstream collagen production. TGF-β in turn, downregulated miR-18a in stellate cells, indicating that there may be a balance in miR-18a expression and TGF-β pathway signaling. Our study highlights the careful research in analyzing whole tissue expression to understand more clearly the functional role of miRNAs. miR-18a can function as a prognostic indicator during fibrosis; increased miR-18a could downregulate TGF-β pathway signaling and protect against a severe fibrotic phenotype.

4.3 Future study opportunities and strategies

The protection of miR-29a in reversing liver fibrosis even when predominantly hepatocytes are transduced raises many questions and further avenues of research. miR-29a could independently target proteins in both hepatocytes and stellate cells, resulting in regulation of fibrosis or miR-29a could be transferred in a paracrine fashion between the two cell populations. Further research along these lines will identify cell specific effects of miR-29a and validate the use of AAV in clinic.

miR-18a is increased during alcoholic liver fibrosis and it is downregulated by the major pro-fibrotic cytokine TGF-β. These contradictory information could be resolved by isolating individual stellate cells in fibrotic livers and analyzing the expression of miR-18a in these cells. Overall increase in miR-18a in whole tissue could also be explained by increase in the total number of stellate cells which can also be verified by isolation of stellate cells from rodent livers. Additionally, delineating the specific effects of CTGF downregulation and Smad2 downregulation on the functional fibrotic phenotypes of stellate cell migration and collagen protein production can be useful in furthering the understanding of miR-18a's protective role in fibrosis.

Liver fibrosis is a significant clinical problem with no existing therapy to target the underlying fibrotic mechanisms. miRNAs can play a powerful role in treating diseases since they target many proteins and have showed no adverse phenotype so far with overexpression. We have contributed to the understanding of miRNA biology with the study of 2 different protective miRNAs, miR-29a and miR-18a, which will support the existing body of evidence of miRNA as therapeutics. It is our hope that we have begun the first step of developing successful treatment strategies for liver fibrosis.

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Appendix 1. Primers used for cloning.

Sequence	F primer	R primer
GFP	CCACTACCTGAGCACCCAGTC	TCCAGCAGGACCATGTGATC
DGCR8	CCATCAGGCAATGGCTCTGT	TGCAGGATGTTTTTTGTGTTCTG
Smad2 human	GAAAACAGGACGATTAGATGAGC	GACCTGGTTTGTTCAGAGAAGC
Smad4 human	AATGCTACCAGCACTGCCAA	ATCCATTCTGCTGCTGTCCT
Col1a1 human	CAAGAGGAAGGCCAAGTCGAGG	CGTTGTCGCAGACGCAGAT
CTGF human	CCTGGTCCAGACCACAGAGT	TGGAGATTTTGGGAGTACGG
18A-PAAV 1	ATGGGCCGGCCGCCTGCTGATGTTGAG TGCTTTTTGTTCT	GCT GGCCGGCC TTAAAACACCTATATACTTGCTTGGCTTG
18A-PAAV 2	ATGGGCCGGCC AGCTGCCTCGGGAAGCCAAG	GCTGGCCGGCCAAACAAAAACTATTAAAA CACCTATATACTTG
18A-PAAV 3	ATGGGCCGGCC ATCTACTGCAGTGAAGGCACTTG	GCT GGCCGGCC GTGCAACTATGCAAAACTAACAGAG
Primary 18a rat 1	GTGAAGGCACTTGTGGCATTG	AGAAGGAGCACTTAGGGCAGT
Primary 18a rat 2	TTGAAGTCTGTCACAGGGTTTGC	TTCAGTCCCTCCCAAGACGAT

Curriculum vitae

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- Thesis: "Protective roles of mir-29a and mir-18a in liver fibrosis"

B.Tech. Biotechnology. Anna University. Coimbatore, India.
- May, 2006.
- GPA: 3.77/4. *Magna Cum Laude.*

Research Experience:

2006-present. John Hopkins University School of Medicine. Baltimore, MD.
- Thesis advisor: Dr. Daniel S. Warren
- The goal of my research was to identify the roles of miR-29a and miR-18a in liver fibrosis
- Developed a therapeutic model for delivery of miR-29a and successfully reversed liver fibrosis in mice
- Identified that miR-18a is up regulated in fibrotic livers in fibroblasts and characterized its role in modulating fibrosis by down regulation of TGF-β pathway.

2006. Tata Institute of Fundamental Research. Mumbai, India
- Studied peptide chemistry using LC-MS

Summer 2005. Center for DNA Fingerprinting and Diagnosis. Hyderabad, India
- Identified sex-specific differential gene expression in silkworms

Work, Teaching, & Leadership Experience:

2008. The Johns Hopkins School of Medicine. Baltimore, MD
- Teaching assistantship: Fundamentals of Genetics.

2007. The Johns Hopkins University. Baltimore, MD
- Organizer, Classical Indian dance group, JHUMOOR
- Event Manager, JHUMOOR

2005. Anna University. Coimbatore, India
- Executive, Students Union

- Planner of technical symposiums and cultural events

2004. Anna University. Coimbatore, India

- Joint secretary, Biotechnology Association

Awards & Honors:

2006- Gold medal for Excellence in Biotechnology; cash award
2005- Indian Academy of Sciences Summer Fellow
2002- Merit Scholarship from the Government of India

Peer-reviewed publications:

Knabel M*, **Ramachandran K***,Karhadkar S, Hwang H, Creamer T, Chivukula RR, Sheikh F, Clark KR, Torbenson M, Montgomery RA, Cameron AM, Mendell JT, Warren DS. *Systemic delivery of scAAV8-encoded miR-29a ameliorates hepatic fibrosis in carbon tetrachloride-treated mice* Molecular Therapy. 2013 (under review) (* co-authors)

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Presentations:

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Ramachandran K, Chau NB, Irusta PM, Hardwick MJ. *Role of Bcl-xl interacting protein Aven in transcription,* Keystone Cell Death Pathways conference, Canada. 03/2009. Poster

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